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Announcement

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Cytological Studies of Tumors. XIV. Isolation of Single-Cell Clones from a Mixed-Cell Tumor of the Rat [1, 2, 3]

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The concept of stemline-cells from which tumors are propagated was developed by Makino, in collaboration with his co-workers (*1–5*) on the basis of cytological studies of several ascites tumors in rats, including morphological and statistical analyses of the tumor cells. This concept was borne out mainly by the continued existence of populations of tumor cells that were characterized by a high frequency of occurrence, definite chromosome patterns persistent through serial transfers, and regular mitotic behavior. Furthermore, it was confirmed that the individuality of the stemline-cells remained unchanged through heteroplastic transplant experiments and through treatment with chemicals. Recently, Levan and Hauschka (*6*, *7*) and Yosida (*8*, *9*) reported the occurrence of a similar chromosome feature in ascites tumors of mice.

The investigation reported herein was a re-examination of the stem-cell hypothesis through experiments conducted with single-cell inoculations, and compared the chromosomes in single-cell clones with those in the stock tumor.[3] For the critical single-cell isolation, the Hirosaki sarcoma was chosen as favorable material because it is characterized by several stemline-cells, as shown by Makino and Kanô (*2*).

Materials and Methods

In this former paper published by Makino and Kanô (*2*), it was stated that the Hirosaki sarcoma was characterized by the presence of five stemline-cells—distinguishable from one another by 1, 2, 3, 4, and 5V-shaped chromosomes of outstandingly large size. However, in the later generations a striking alteration took place in the frequency of the stem-cells resulting in the complete disappearance of the 4V- and 5V-type cells.

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[2] Contribution No. 313 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan. Supported by a grant from the Scientific Research Fund of the Ministry of Education.

[3] The outlines of the experiments were communicated to the 12th General Meeting of the Japanese Cancer Association at Tokyo (Gann 44: 144, April 1953), and the 13th meeting at Nagoya (April 1954).

[4] Grateful acknowledgment is made here to Dr. T. Tanaka for his stimulating discussion and technical assistance

In the present study another line of the Hirosaki sarcoma was used as material. In an early transplant generation (the 22nd) this line showed the frequency distribution of five types of stem-cells, as shown in table 1–A, while in a later generation (the 73rd) their frequency distribution changed considerably (table 1–B); the 2V- and 3V-type cells appeared at 40.3 percent and 44.0 percent, respectively, with a disappearance of the 5V-type cells. At the time of the present study the chromosome feature of this line was as in the 73rd generation.

TABLE 1.—*Frequency of occurrence of the five cell types in the 22nd and 73rd transplant generations*

Type of cells	Frequency	
	A (22nd generation) (*percent*)	B (73rd generation) (*percent*)
1V	2. 9	5. 7
2V	17. 4	40. 3
3V	20. 3	44. 0
4V	50. 7	10. 0
5V	8. 7	0

The procedure for single-cell isolation followed the technique devised by Ishibashi (*10*) and Hosokawa (*11*, *12*) for the Yoshida sarcoma. The tumor ascites containing tumor cells was removed from tumor-bearing rats on the 5th day after transplantation, and immediately diluted 1:20,000 with diluting fluid.[5] With the use of a sterilized glass pipette the diluted tumor ascites was dropped on clean coverslips (24″×50″), about 20 droplets for each slip. The coverslip, thus prepared, was set on the micromanipulator (Zeiss), and at a magnification of 200 ×, a microscopic droplet containing a single, or a few cells, was sucked up in a micropipette. Examination was made under the microscope to confirm the number of cells thus drawn into the micropipette. After the addition of a small amount of diluting fluid,[6] the cells (of known number determined in the manner described above) were then injected from the micropipette into the peritoneal cavity of the rat. In addition, the droplets found to be cell-free were injected to obtain control data. Albino rats weighing from 55 to 120 grams and derived from several strains, were used in these studies. The inoculation of a single cell was most commonly successful in young rats from which the transmissible strain of the Hirosaki sarcoma had its origin.

The study of the chromosomes was carried out by the use of smear preparations of the ascites tumor that were stained with acetic gentian

[5] In the early experiments normal saline alone was used as the diluting medium with unsuccessful results, probably because the cells had been injured. The diluting fluid used here was prepared as follows: 15 to 20 cc. of normal saline (0.85% NaCl solution) was injected with a sterilized syringe into the peritoneal cavity of rats weighing 100 to 120 grams. After 15 minutes, the ascites fluid was drawn from operated rats into a sterilized syringe, and spun at 3,000 r.p.m. for 1.5 hours. Centrifugal supernatant, thus prepared, was used as the diluter, after examination under the microscope to prove that the supernatant was absolutely free of cells.

[6] Without adding a small quantity of diluting fluid, the cells were too fragile to handle for inoculation.

violet and acetic dahlia. For details of the technique reference should be made to previous papers (*1*, *2*).

Observations

Results of Inoculations with Isolated Tumor Cells

The general scheme of the experiments and results of inoculations with isolated tumor cells are shown in table 2. Inoculations with isolated cells were made in a total of 58 rats of which 7 developed the ascites tumor. Details are as follows:

1) *Five-cell inoculation.*—Inoculations with five tumor cells were made in two experiments. One of the two strain G rats injected showed successful inoculation and died with the ascites sarcoma 18 days after inoculation (table 2).

2) *Three-cell inoculation.*—A strain Wp rat that received three isolated tumor cells failed to produce the disease.

3) *Two-cell inoculation.*—Four rats were injected with two isolated tumor cells. Successful injections were made in two rats (one each of the T and W strains) and they died with the ascites sarcoma on the 16th and 25th day after inoculation (table 2). The ascites sarcoma developed in the strain W rat was transmitted by intraperitoneal injection of the tumor for eleven generations.

TABLE 2.—*Summary of experiments*

Material injected (*cells per rat*)	Rats used			Number of successful injections	Host survival time (*days*)
	Strain*	Number of rats injected	Body weight (*gm.*)		
5	Wp	1(♀)	85	0	—
5	G	1(♂)	98	1(♂)	18
3	Wp	1(♀)	80	0	—
2	Wp	1(♂)	85	0	—
2	W	2(♀, ♂)	95, 120	1(♀)	25
2	T	1(♀)	60	1(♀)	16
1	Wp	11(♀6, ♂5)	60–100	0	—
1	WX	4(♂)	90–100	0	—
1	W	12(♀4, ♂8)	75–80	0	—
1	G	7(♀3, ♂4)	90–120	0	—
1	T	17(♀8, ♂9)	55–80	4(♀1, ♂3)	17–25
Total		58		7	
Cell-free material	W&T	12(♀7, ♂5)	60–120	0	—

*Details of the rat strains are as follows: W=Wistar rats purely inbred. WX=a strain originated from the cross of the Wistar rats and the Yamashita rats. Wp=a strain derived from the Wistar rats producing large litters. G=rats from Gifu—not purely inbred albinos. T=rats from Tokyo—not purely inbred albinos.

4) *A single-cell inoculation.*—Inoculations with single tumor-cells were made in 51 rats, which included derivatives from five different strains. Only 4 of the injected rats were successfully inoculated; they developed

the ascites sarcoma and died with the disease after from 17 to 25 days (table 2). It is of interest to note that the 4 rats that received successful inoculation with a single cell were all derivatives of the T strain, a strain that was also specially susceptible to the transmission of the stock Hirosaki sarcoma. Detailed data on these single-cell derivatives are presented in table 3.

TABLE 3.—*Data of the successful injection in single-cell inoculation of 4 rats*

Material injected (*cell per rat*)	Rats used			Host survival time (*days*)	Condition of tumor ascites at first appearance	Days of tumor cell proliferation after injection	Subline
	Strain	Sex	Body weight (*gm.*)				
Single	T	♂	75	25	Bloody	12	B-H
Single	T	♀	65	17	.....	7	C-H
Single	T	♂	60	21	Bloody	7	D-H
Single	T	♂	65	22	.....	6	E-H

The percentage of successful inoculation with single cells was very low. Only 4 of the 51 rats injected developed the ascites sarcoma. The difficulty of transmitting with single cells may be due to the fact that the cells used for inoculation may be either injured or lost during the process of micromanipulation.

The ascites tumors developed by the introduction of a single tumor-cell into each of the 4 rats were transmissible from rat to rat by intraperitoneal injection. Four tumor sublines were established. They are referred to as the B-H, C-H, D-H, and E-H sarcoma, respectively. Obviously, each was a derivative from the progeny of one original cell isolated from the stock Hirosaki sarcoma. Generally the essential nature of these sublines, as well as their type of the disease, was identical to those of the stock tumor from which the single cell of origin was derived (figs. 1 to 3).

Of the four sublines of ascites tumors, the B-H and E-H sarcomas failed to succeed in transmission after the 24th and 2nd transplant generation, respectively. The C-H sarcoma has been successively transmitted for 74 generations and the D-H for 40 (March 1954).

5) *Inoculation with cell-free material.*—Droplets found to be cell-free under the microscope were sucked up into the micropipette and injected intraperitoneally. Twelve rats that received cell-free material completely failed to develop the disease at all.

Chromosomes in the Four Single-Cell Sublines

As described above, four single-cell sublines were derived from the Hirosaki sarcoma through microisolation. Prime interest in the present study was directed toward the chromosome patterns in these single-cell sublines. The study of chromosomes was made with smear preparations of the tumor stained with acetic gentian violet. The material for the chromosome study was sampled from more than 5 tumor-bearing rats in each subline, except E-H, through their whole or partial transplant genera-

tions. The numerical data given in table 4 indicate average values for each subline.

TABLE 4.—*Cytologic data of four single-cell sublines*

Subline	Cell type	Chromosome No.	Frequency of cells (*percent*)		Transplant generation	Average survival time * (*days*)
B-H	3V	37 (36–39)†	3V	79.4 (95)‡	24	8.7
			aberrant	20.6 (25)		
C-H	2V	39 (37–41)	2V	90.0 (90)	74	9.4
			aberrant	10.0 (10)§		
D-H	3V	37 (35–39)	3V	79.2 (67)	40	9.1
			aberrant	20.8 (18)‖		
E-H	2V	38 (36–40)	2V	81.3 (48)	2	—
			aberrant	18.7 (11)		

*Average life span of the stock Hirosaki sarcoma was 11.2 days.
†Numerals in parentheses denote the range of variation in chromosome number.
‡Numerals in parentheses indicate the number of cells observed.
§Of this, 6.5 percent was represented by the cells showing extrusion of large V-chromosomes from the equatorial plate.
‖Of this, 6.1 percent was represented by the cells showing extrusion of large V's from the equatorial plate

1) *B-H sarcoma.*—The present investigation revealed that, as in other ascites tumors (*1*, *2*), there was present a distinct population of tumor cells, which occurred at a high frequency and were characterized by a well-balanced subdiploid complex of chromosomes. These cells multiplied in a regular mitotic manner showing usual chromosome behavior. Considering the evidence observed in other ascites sarcomas, there is no doubt that these cells with well-balanced chromosomes are the primary contributors to the growth of this tumor. The chromosomes of these cells in material sampled from 5 tumor-bearing rats at the 1st, 3rd, 5th, 10th, and 23rd transplant generations, were analyzed by comparing them to those of the Hirosaki sarcoma from which this subline was descended.

The chromosome number observed in these cells exhibited a rather narrow range of variation of from 36 to 39, the most frequent number being 37. The chromosome complex consisted of two distinct groups of chromosomes; namely, the group containing certain numbers of rod-shaped elements and that made up of certain numbers of J- and V-shaped ones. Analysis of the morphology of the chromosomes in a number of cells revealed that the chromosomal constitution was represented by 20 to 24 rod-shaped chromosomes and 16 to 19 J- and V-shaped ones, and that every complex was remarkable in containing prominent V-shaped chromosomes of outstandingly large size which were *always three* in number (figs. 4 to 6, and 16). After a comparison of the chromosomes between the present sarcoma and the stock tumor, Hirosaki sarcoma, it was assumed that the tumor cells of this subline corresponded to the 3V-type cells of the Hirosaki sarcoma because of the fair conformity in their chromosome constitution. Since the B-H sarcoma is a single-cell derivative of the Hirosaki sarcoma, these tumor cells are those derived from the progeny of one original 3V-type cell isolated from the Hirosaki sarcoma.

The numerical study indicated that the 3V-type cells were predominant in occurrence, appearing in about 80 percent of the tumor cells observed (table 4). In addition the aberrant cells, showing various mitotic abnormalities, occurred in about 20 percent. It is certain that these aberrant cells are all descendants from one original 3V-type cell. Stickiness of chromosomes, deformation of chromosomes into unusual-shaped bodies, nondisjunction and lagging of chromosomes, appearance of chromosome bridges at ana-telophase, irregular distribution of chromosomes at anaphase, displacement of chromosomes at metaphase, extrusion of some chromosomes from the equatorial plate, and multipolar mitosis were remarkable mitotic abnormalities observed here. Particularly striking were extrusion of V-shaped elements of large size from the metaphase plate and their lagging at anaphase following nondisjunction (figs. 22 to 24). The aberration of this nature resulted in occasional production of cells showing small numbers of large V-shaped chromosomes.

2) *C-H sarcoma.*—Like the former tumor, the C-H sarcoma showed a population of cells with a well-balanced subdiploid complex of chromosomes. The chromosomes of these tumor cells were analyzed for comparison of their morphology with that of the chromosomes of the Hirosaki sarcoma, whose cell was used to originate this tumor. This analysis was based on the material obtained from 8 tumor-bearing rats at the 1st, 3rd, 5th, 10th, 25th, 45th, 60th, and 70th transplant generations. The number of chromosomes of these cells varied from 37 to 41, with the modal number of 39. The cells were provided with certain numbers of rod-, V- and J-shaped chromosomes of varying sizes. Closer investigation made it clear that the chromosome complex was made up of from 23 to 26 rod-shaped elements and from 14 to 17 J- and V-shaped ones. Furthermore, every cell was characterized by having, without exception, outstandingly large V-shaped chromosomes which were *always two* in number (figs. 7 to 9, and 17 and 18). Here, a comparison revealed that the chromosomes of these tumor cells showed a striking similarity to those of the 2V-type cells of the Hirosaki sarcoma. Since the C-H sarcoma is a single-cell clone derived from the Hirosaki sarcoma, these 2V-type cells are evidently descendants from the progeny of one original 2V-type cell introduced from the stock tumor through inoculation.

The numerical study showed that in this tumor the 2V-type cells existed in an exceptionally high frequency, 90 percent of the cells being of this type (table 4). Aberrant cells of various kinds, representing 10 percent of the total number, were undoubtedly derivatives from the progeny of one original 2V-type cell. As in the former single-cell subline, extrusion of large V-shaped chromosomes from the equatorial plate and their lagging due to nondisjunction were observable as remarkable abnormalities, and occurred in 6.5 percent of the cells. It is highly probable that an abnormality of this nature caused the occasional appearance of cells that contained one large B-element.

3) *D-H sarcoma.*—Also in this subline the occurrence of a definite population of tumor cells was confirmed on the basis of the characteristic,

well-balanced subdiploid complex of chromosomes in the cells and the exceedingly high rate at which such a complex appeared. The number of chromosomes in these tumor cells was found to vary from 35 to 39, with 37 as the most common number.

Investigation of the chromosomes in many dividing cells observed in the samples from 6 tumor-bearing rats at the 1st, 5th, 10th, 20th, 30th, and 40th transplant generations, and comparison of these cells with those of the Hirosaki sarcoma, made it clear that the tumor stem-cells of this sarcoma corresponded to the 3V-type cells of the stock tumor (figs. 10 to 12, and 19). Every cell was characterized by the constant presence of *three* V-shaped chromosomes of prominently large size. It was therefore concluded that the D-H sarcoma had its origin in a single-cell inoculation of the 3V-type cell isolated from the Hirosaki sarcoma.

Tumor cells of this subline having three prominent V-shaped chromosomes always predominated in occurrence with a frequency as high as about 80 percent. In addition were cells with various abnormalities appearing with a frequency of about 20 percent (table 4). Extrusion of large V-elements from the metaphase plate and their lagging at anaphase migration were observed in about 6 percent. Occasional appearance of cells showing small numbers of large V-elements was explicable as the result of the abnormalities of this nature.

4) *E-H sarcoma.*—As in the former subline there occurred in this tumor a population of tumor cells, which were characterized by a well-balanced subdiploid complex of chromosomes and which occurred with extremely high frequency. They exhibited a variation of chromosome number ranging from 36 to 40, with the modal number of 38. Morphological analysis of the chromosomes in these cells based on the material sampled from 2 tumor-bearing rats at the 1st and 2nd transplant generations indicated that they corresponded to the 2V-type cells of the Hirosaki sarcoma, on account of the existence of prominently large V-shaped chromosomes, *always two* in number, together with the characteristic other components (figs. 13 to 15, 20 and 21). This complex of chromosomes occurred in about 80 percent of the cells, whereas abnormal mitoses occurred in the remaining 20 percent.

Chromosomes in a Subline Derived Through a Two-Cell Inoculation

It was noted above that of 4 rats that received two-cell inoculations, 2 rats, derivatives of strains W and T, developed the ascites sarcoma and died 25 and 16 days after injection (table 2). The successive transmissions were successful in the tumor developed in the W-rat for eleven generations, the host dying on an average of 9 days following inoculation. The following description refers to the chromosomes principally investigated in this sarcoma. In this study sampling was made from 6 tumor-bearing rats at the 1st, 2nd, 3rd, 5th, 7th and 10th transplant generations.

After investigation it was clear that there were two distinct tumor-cell populations present in this tumor. Both had a high frequency of occur-

rence but had different complexes of chromosomes. The one population was characterized by the tumor cells having two V-shaped chromosomes of outstandingly large size together with rod-, J- and V-shaped ones of varying sizes; whereas the other population was characterized by cells possessing three such large V's with other elements of similar nature. Since this sarcoma was a descendant from the Hirosaki sarcoma through a two-cell inoculation, it was evident that the occurrence of both kinds of cells with 2V's and 3V's was due to implantation of both 2V- and 3V-type cells of the stock sarcoma.

It was shown by a numerical investigation that the frequencies in occurrence of the two types of cells were 55.1 percent for the 2V-type cells and 38.5 percent for the 3V-type cells, together with aberrant cells with a frequency of 6.4 percent. Among the latter were found a few cells that showed unusual numbers of the large V-chromosomes. Most probably, they are explicable as the result of extrusion of the V-elements from the metaphase plate and their nondisjunction at anaphase, a feature which was one of the remarkable abnormalities occurring in other sublines of sarcomas as mentioned above.

The numerical variations of chromosomes exhibited by the two types of cells were from 36 to 40 for the 2V-type cell and from 35 to 40 for the 3V-type cell, the modal number being 38 for both cells. These data closely approximate those formerly obtained for the 2V-type and 3V-type cells, respectively (table 4).

Chromosomes in the Two Tumor Derivatives Produced by a Simultaneous Inoculation with Two Isolated Cells

Two tumor derivatives were produced, after many attempts, by a simultaneous inoculation with two isolated cells, one from the B-H sarcoma and the other from the C-H sarcoma, with a technical procedure similar to that already described. The survival time of the host was 7 and 13 days for the two derivatives, respectively. The chromosomes observed in these sublines are described here.

As was to be expected, the two tumor derivatives under consideration here were characterized by the existence of two distinct populations of tumor cells, both having a high frequency of occurrence but differing in their chromosomal constitutions. Cytological investigation revealed that the two populations were represented by 2V- and 3V-type cells, respectively; the former cells were characterized by two V-shaped chromosomes of remarkably large size, and the latter by three such V's. The cells of the 2V-type occurred with a frequency of 67.6 percent in the one derivative and 51.4 percent in the other, while those of the 3V-type occurred with a frequency of 25.6 percent in the one and 37.8 percent in the other. The chromosomes varied in number, from 36 to 40 for 2V-type cells and from 35 to 40 for 3V-type cells, with the modal number of 38 for both types. The numerical data presented here nearly approach those shown by the former tumor derived from two-cell inoculation.

Discussion and Conclusion

Many experiments have been done to determine the number of cells necessary for the transmission of malignant tumors of mice. It was generally accepted that more than a hundred thousand cells were necessary to transmit mouse carcinomas and sarcomas, until the first successful transmission of mouse leukemia with a single leukemic cell was attained by Furth and Kahn (*13*). Following the successful attempt of the single-cell transplantation in the Yoshida rat sarcoma by Ishibashi (*10*), with a simple technique, Hosokawa (*11*, *12*) conducted similar experiments and showed that the tumor could be transmitted with a single tumor-cell inoculation, and that cell-free material was not capable of producing the disease. Sato (*14*) investigated the chromosomes of the tumor cells in the tumor clones produced by Hosokawa (*11*, *12*) after injection of a single cell. He failed to observe distinct cytological differences between the clones and their stock tumors and questioned the presence of the stem line of tumor cells as primary contributors to the growth of the tumor.

Cytological investigations involving both morphological and statistical analyses of chromosomes by Makino and his co-workers (*1–5*) have revealed the existence of the stem line (or lines) of tumor cells, on the basis of the occurrence of populations of tumor cells characterized by a high frequency, definite ideograms and regular mitotic behavior. Need for further confirmation of the concept of stemline-cells suggested a re-examination through comparison of chromosomes in the original stock tumor and those in single-cell clones derived through isolation experiments. For the critical single-cell isolation in this work, the Hirosaki sarcoma was chosen as interesting material because the latter contains several stemline-cells characterized by different chromosome constitutions (*2*).

Positive results were obtained for single-cell inoculations with the Hirosaki sarcoma in 4 of 51 trials in this study (table 2). The fact that positive results were obtained in only a small percentage of inoculated animals may be explained by supposing that most of the isolated cells were not viable, that they were lost or injured during the process of manipulation, or that they did not have the stem cell's capacity to propagate autonomously. It may be of significance that four successful transplantations were all made in the rats of strain T, which were highly susceptible to the stock tumor.

The tumors that developed in the 4 rats receiving single-cell inoculations were able to give rise to four distinct sublines capable of serial transmissions (table 4).

Cytological investigation with these four single-cell derivatives revealed that each tumor clone was characterized by a neoplastic population provided with tumor cells of a single-cell type; two of the four sublines were found to be 3V-type cell derivatives since they were characterized by the population of 3V-type cells, while the other two tumors were regarded as 2V-type cell derivatives because of the population consisting of 2V-type cells. Probably, both 2V-type and 3V-type cells were more

predominantly selective in isolation than the other two kinds of cells, 1V- and 4V-type cells, on account of their prevalent proportion of occurrence in the stock tumor (table 1).

It was of interest to note that the single-cell tumor clones isolated from the original stock tumor of a mixed-cell type showed a malignant growth, as well as the characteristics of disease, more or less comparable to the stock tumor.

It was shown after the chromosome analysis that each single-cell derivative was characterized by a stem-cell lineage with characteristic chromosome pattern. The variation in the chromosome number occurred within a narrower range in the single-cell derivatives than in the stock tumor. The frequent chromosome-number modes for both cell-types in the single-cell clones were found to be nearly identical with those found for them in the original stock tumor of the mixed-cell type. In comparison with the stock tumor, the cytological homogeneity of these derivatives is rather remarkable. It is impressive that the independent cytological patterns of tumor cells has persisted as such for over 70 and 40 serial transfers for the 2V-type cell and 3V-type cell derivatives, respectively.

Cytological study made in a tumor derivative established with two-cell inoculation revealed the occurrence of two different neoplastic populations provided with 2V- and 3V-type cells, respectively. Both kinds of cells appeared with definite frequencies in each population. The condition had persisted for eleven successive generations when the transmission failed due to lack of experimental animals. Further investigation on the tumor derived through a simultaneous inoculation with two different cells, one isolated from the 2V-type cell clone and the other from the 3V-type cell clone, also revealed the appearance of two cell-populations, one represented by the 2V-type cells and the other by the 3V-type cells. It is interesting that the cells of both types appeared at a definite rate of occurrence, with rather identical value to that found in the former two-cell clone.

Thus, the isolation experiments with the Hirosaki sarcoma in this study resulted in the production of pure single-cell clones isolated from the original stock tumor of a mixed-cell type. Each clone was characterized by a stemline chromosome pattern which is structurally distinct from those of the other clones and has persisted without visible shift during certain transplant generations.

All the cytological features derived from the isolation experiments in this study seem to have greatly strengthened the concept of stemline-cells as progenitors of the neoplastic population. Recently, the reality of the stem-cell concept was strongly supported by Hauschka (*15*, *16*) on the basis of the results of his experiments on single-cell inoculation of ascites tumors in mice. His results showed the possibility of establishing pure single-cell clones more uniform than the original tumor, and coincide with the findings of this study.

Summary

For the purpose of a re-examination of the concept of the stem-cell from which the tumor develops, experiments of single-cell inoculation after

microisolation were made with the Hirosaki sarcoma, an ascites tumor of mixed stem lines in rats. Single-cell inoculation was successful in 4 of 51 trials, and established the isolation of pure single-cell clones from the original stock tumor. Each single-cell clone was characterized by a stem-line chromosome pattern, which was structurally distinguishable from those of the others and which persisted without visible shift during certain serial transfers. Each clone showed a malignant growth more or less comparable with the stock tumor. The results of the isolation experiments strengthened the stem-cell concept.

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PLATE 69

Tumor cells of the stock tumor and single-cell clones, on the 4th day after transplantation. × *200*

FIGURE 1.—Hirosaki sarcoma (stock tumor).

FIGURE 2.—C-H sarcoma (2V-type cell clone).

FIGURE 3.—D-H sarcoma (3V-type cell clone).

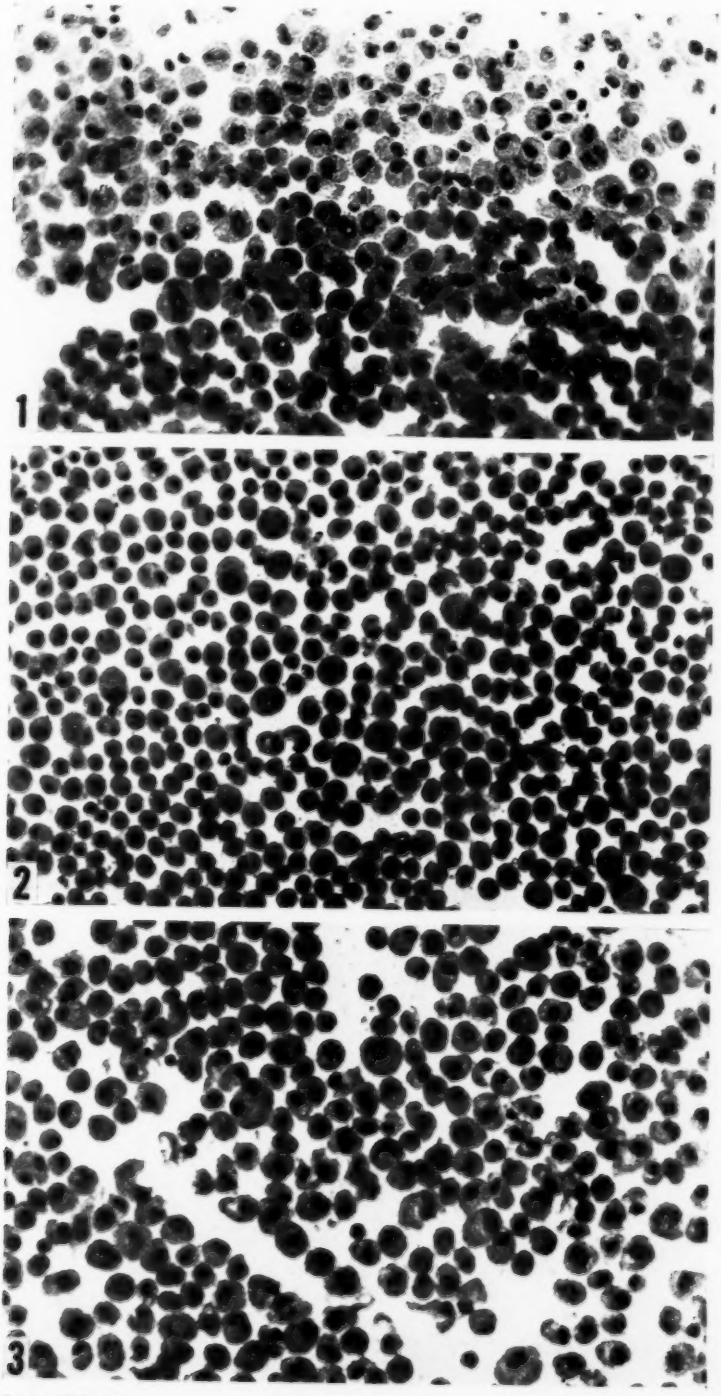

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PLATE 70

Metaphasic chromosomes of stemline-cells of single-cell clones. × *3,800*

FIGURES 4 to 6.—B-H sarcoma (3V-type cell clone).

FIGURES 7 to 9.—C-H sarcoma (2V-type cell clone).

FIGURES 10 to 12.—D-H sarcoma (3V-type cell clone).

FIGURES 13 to 15.—E-H sarcoma (2V-type cell clone).

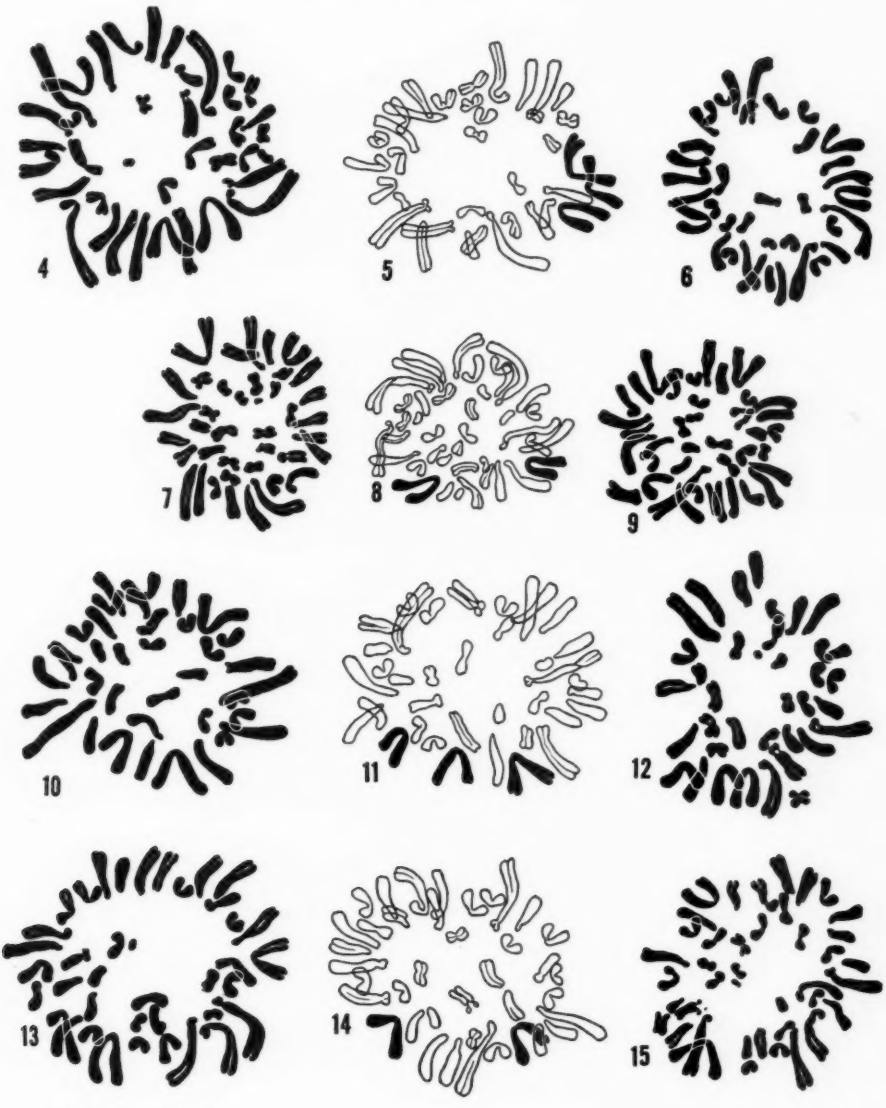
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PLATE 71

Photomicrographs of chromosomes of tumor stem-cells from single-cell clones. × *2,000*

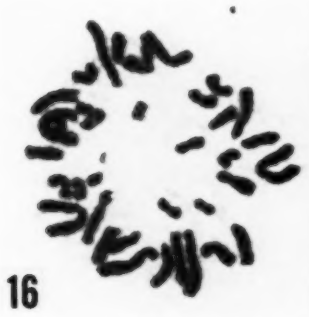
FIGURE 16.—B-H sarcoma (3V-type cell clone).

FIGURES 17 and 18.—C-H sarcoma (2V-type cell clone).

FIGURE 19.—D-H sarcoma (3V-type cell clone).

FIGURES 20 and 21.—E-H sarcoma (2V-type cell clone).

FIGURES 22 to 24.—These figures show the extrusion of a large V-shaped chromosome at metaphase and anaphase. From B-H sarcoma. × 2,000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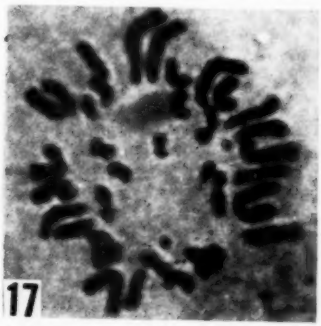
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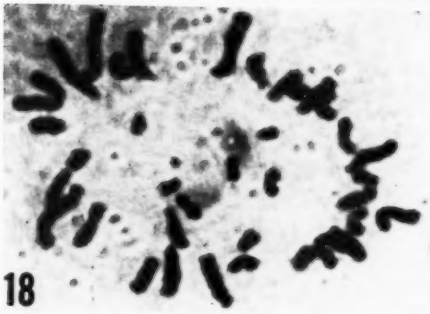
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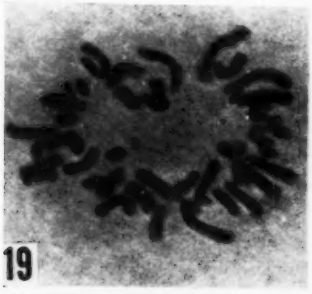
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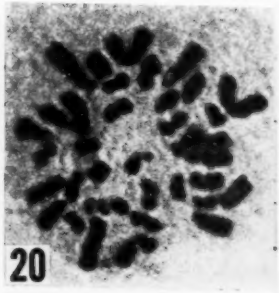
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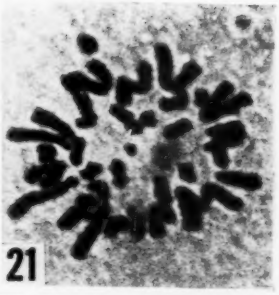
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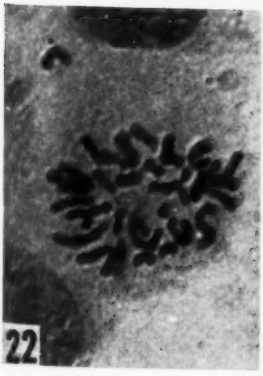
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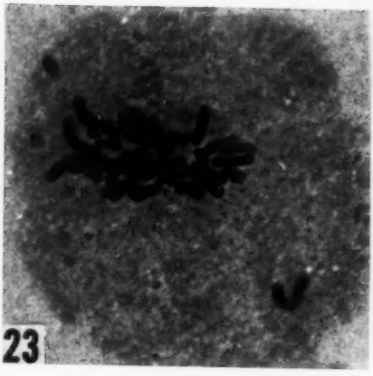
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827933—55——5

Congenital Polycystic Disease of the Kidney Occurring in Mice[1,2]

BARBARA M. RUPPLE,[3] *Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and the Department of Pathology, College of Medicine, Ohio State University, Columbus, Ohio*

Congenital polycystic disease of the kidney, although relatively common in man, has not been known to appear spontaneously in small laboratory animals. Considering the vast number of mice and other laboratory animals that have been autopsied in the course of years in many laboratories, it is noteworthy that naturally occurring congenital cystic disease of the kidney has heretofore never been recorded. Cystic renal lesions have been observed in rats and mice; in most instances where histologic studies are lacking, the exact nature of the renal lesion is not clear. The literature contains no confirmed examples of congenital polycystic disease of the kidney occurring spontaneously in small animals.

As described by Anderson (*1*) congenital polycystic disease of the kidney (in man) is a hereditary maldevelopment. One or both kidneys may be involved by extremely numerous cysts of varying and often large size. The condition is present at birth, and absence of sufficient functioning renal tissue may result in death at that time or within the next few years (in man). The involved kidneys may be moderately or enormously enlarged. Enlargement is due to increase in size of individual cysts, rather than to increase in their number. The kidneys have a knobby or irregular outline. The cysts are lined by cuboidal or, more commonly, flattened epithelium. The hereditary tendency of this disease in man has long been established by numerous reports of its occurrence in several generations in one family.

Many theories have been proposed regarding the mechanism by which polycystic kidneys develop in the course of embryonic life. There are two basic concepts: 1) Retention of ontogenetic structures. In the normal course of development cysts are formed from ureteral bud elements; later these structures atrophy; however, theoretically, the cystic

[1] Received for publication July 27, 1954.

[2] This investigation was supported in part by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council, and by research grants, C1073 (Jackson Laboratory) and C1623 (Ohio State University) from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service. It was also aided by a grant to the Jackson Laboratory from the Jane Coffin Childs Memorial Fund for Medical Research.

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structure may persist and give rise to polycystic disease (*2*). 2) Non-union of renal tubular elements. It is thought that in polycystic disease the distal convoluted tubules may fail to unite with the collecting tubules; as a result, cystic dilatation of nephronal elements may occur (*3*).

In addition to these two basic theories, other mechanisms have been proposed. Staemler (*4*), after a discussion of the structure and growth involved in polycystic disease, interpreted this renal lesion as a cystadenofibroma. Brigidi and Severi (*5*) considered polycystic kidney a true neoplastic lesion. Problems regarding the genesis of this disease and its neoplastic nature remain uncertain and merit investigation. Studies of the pathologic processes of polycystic disease in the past have been based upon the analysis of episodic cases as they occur in man. Resolution of this problem would be facilitated if a study could be made using cystic kidneys occurring in laboratory animals. In the present work, polycystic kidney was discovered in a colony of miscellaneous albino mice being inbred by brother × sister matings and selected for mammary gland tumors; the description of this finding forms the basis of the present report.

In the literature there have been described in laboratory animals various cystic lesions of the kidney—some spontaneous, some induced. In no instance is a lesion described which conforms structurally and biologically to the spontaneous familial lesion occurring in man. Gorer (*6*) described in old mice a cystic renal lesion arising in obstructed collecting tubules following necrosis of renal papillae. Dunn (*7*) described cystic dilatation of tubules and glomerular capsule associated with renal amyloidosis. Bagg (*8*) described the incidental occurrence of a unilateral multicystic renal lesion in the progeny of irradiated animals; the various renal lesions observed here were thought to be due to an alteration of the germ plasm resulting from irradiation. In 1,817 mice, studied by radiographic technique, three instances of unilateral renal lesion, polycystic in nature, are described. Hain and Robertson (*9*) describe a cystic kidney lesion in rats; the gross description indicates that the renal malformation here was hydronephrosis.

The present work was initiated during the establishment of an inbred strain of albino mice, strain DA (*10*). Attention became focused on members of this strain which were undersized and bore polycystic kidneys. In the ninth generation of brother × sister matings, animals were observed bearing multiple somatic defects. Noted first was the feature of size; runted animals were observed. The affected mice, small at birth, appeared irregularly at first in litters of this strain. These smaller animals were undersized at birth, but in comparison to their litter mates, the retarded body development of the runted animals persisted after periods of 4 and 5 weeks.

Whereas the average newborn mouse is approximately 18 mm. in length, the runted animals were often as small as 10 mm. in length at birth. In a given litter, normal sized mice at 21 days measured 32 mm. In contrast, runted litter mates at this time measured 24 mm. The difference in

relative sizes of the runted and normal litter mates is demonstrated in figures 1 and 2. Figure 1 shows a runt 4 days of age and its normal litter mate.

Although noted sporadically in litters, these runted animals were not autopsied. They were regarded as gestational or nutritional accidents and were given little consideration. The number of runted animals appearing in the colony increased. The consistent occurrence of these anomalous animals became the source of considerable curiosity and an investigation of this growth retardation was initiated.

All runted animals were subsequently autopsied. The kidneys showed striking abnormalities. The runted mice had enlarged, pale, foamy kidneys (fig. 2). In the normal mouse of 4 weeks, the kidney is about 9 mm. long and has a smooth mottled tan and red external surface. The cut surface is gray-red and shows sharply delineated cortex and medulla. The involved kidneys observed in the runted animals varied in size and structure; in some instances the anomalous kidneys were up to 50 percent larger than kidneys of corresponding normal litter mates. The disproportionate enlargement of the kidneys in the runted animals was emphasized by the contrasting difference in body size. The surfaces of the involved kidneys were pale and vesicular (fig. 2). The cut surface showed obliteration of cortico-medullary architecture; the organ resembled a soft oval sponge. The renal papillae, pelvis and lower urinary tract showed no significant changes grossly.

Enlarged foamy kidneys were found only in runted animals; the severity of renal abnormality was not always proportional to the degree of retardation in body growth. The extent of renal involvement varied considerably in different animals. Some of the involved kidneys showed only slight enlargement and pale tan discoloration. Others, considerably enlarged, showed discreet bleblike structures over the entire external surfaces. Runts with abnormal kidneys, observed first in the ninth generation of brother $\times$ sister matings, were found regularly in the next three generations; however, the incidence and severity of the defects decreased with further inbreeding.

In order to confirm the nature of the renal lesions, the organs were studied histologically. Kidneys from the runted animals showed microscopically a wide range of cystic alterations; the degree of histologic change could be closely correlated with the gross appearance of the kidneys.

The histologic section of a kidney taken from a normal sized mouse in the present study is shown in figures 3 and 4. This kidney, normal grossly, presents a well demarcated cortex and medulla; tubules are generally regular; lumens are small and the epithelium is cuboidal. Kidneys from runted animals, pale and only slightly enlarged grossly, showed on histologic study considerable distortion of cortico-medullary features. Cystic structures, occurring singly and in clusters, and varying in number, were present in the cortex (fig. 5); collecting tubules of the medulla were of normal caliber. Vascular elements showed no changes. The renal papillae were intact; there were no degenerative or inflammatory

changes; there was no amyloidosis. The absence of lesions in the renal papillae precludes a mechanism of genesis such as occurs in the formation of cystic changes in kidneys of older mice as described by Gorer and Dunn (*6*, *7*).

Studied in detail, the cortex showed tubules with varying degree of lumenal enlargement. Some convoluted tubules were only slightly enlarged; the height of the cuboidal epithelium was undisturbed (fig. 6). In other instances there was enlargement to frank cyst formation; the lining epithelium was flattened in a degree corresponding to the enlargement of tubular structures.

In the most severely involved kidneys, those which grossly were large, pale and foamy, the natural landmarks of cortex and medulla were obliterated (fig. 7). Here all degrees of tubular enlargement were evident. The larger cysts were lined with compressed flattened epithelium (fig. 8). Glomerular structures were rare in the most severely involved kidneys; few tubular structures of normal caliber remained. Gross and microscopic study of other organs in the runted mice did not show cystic disease at sites other than the kidneys.

Discussion and Summary

In the present work true congenital polycystic disease of the kidney in mice is defined. A lesion such as this, occurring spontaneously in laboratory animals, has not been previously recorded. This congenital malformation of the kidney which evolved during a genetic study in a strain of mice proved to be hereditary. The familial nature of cystic disease of the kidney, well recognized in human pathology, was recapitulated here in this strain of mice. It was apparent that the occurrence of runted mice with polycystic kidneys was not an accidental phenomenon. The incidence tended to follow an orderly pattern and it was evident at an early date that a hereditary factor was involved. By the ninth generation, occurrence of polycystic renal disease in the runted mice was seemingly well established. However, with further inbreeding the incidence of severe involvement decreased.

The curious concurrence of growth retardation and cystic renal disease merits further study. The relationship of chronic renal disease with retardation of body growth—renal dwarfism—is well known clinically in man. In the present study the most severely affected kidneys showed few intact renal elements; accordingly, it is possible that renal insufficiency occurring here may be the basis for the retardation in growth of the mice.

The mechanism of genesis of true congenital polycystic renal disease has been a matter of controversy. Likewise the relationship of this congenital lesion to renal neoplasia remains unsettled. Correlation of renal cystic disease with the process of neoplasia as suggested by Brigidi and Severi (*5*) and Staemler (*4*) was not apparent in the present study. Lesions such as cortical adenomas which occasionally appear in cystic disease of the kidney in man likewise were not apparent in the mouse.

If a strain of mice producing congenital cystic disease of the kidney were to become available, it would provide a valuable approach to the study of polycystic disease, its genesis, and its relationship to neoplasia.

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PLATE 72

FIGURE 1.—A comparison of size of runted and normal mice, four days old, from the same litter. × 3

FIGURE 2.—A normal sized mouse and its runted litter mate showing relative difference in body size and the appearance grossly of congenital polycystic disease of the kidney in an undersized mouse. × 3

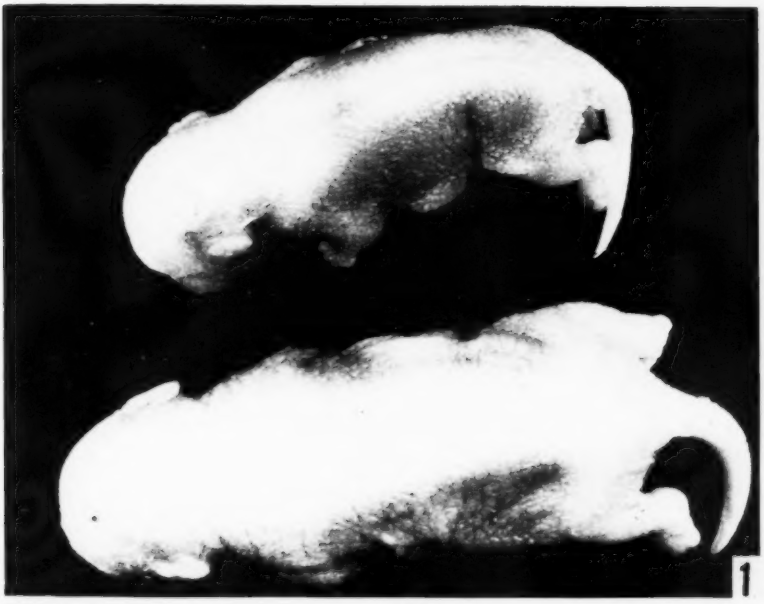

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PLATE 73

FIGURE 3.—Kidney from a mouse having a normal body size and grossly normal kidneys; mouse from the strain in the present study. × 25

FIGURE 4.—Histologic appearance of a kidney from a mouse of normal body size and grossly normal kidneys; mouse from the strain in the present study. × 150

FIGURE 5.—Cystic structures in the cortex of a mouse kidney which grossly showed only moderate alteration. × 25

FIGURE 6.—Polycystic disease of the kidney in mice showing moderate degree of change. × 150

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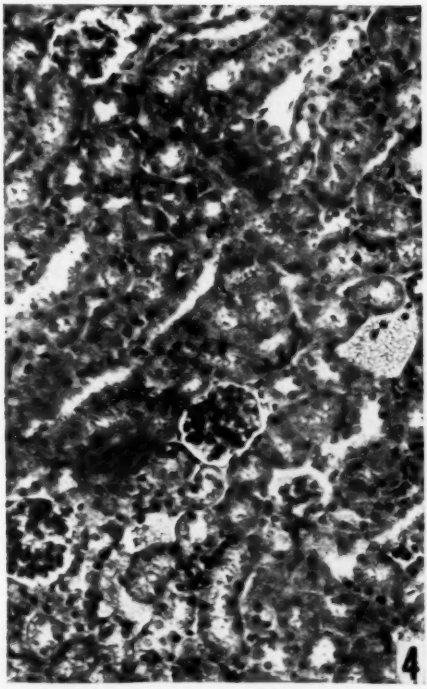

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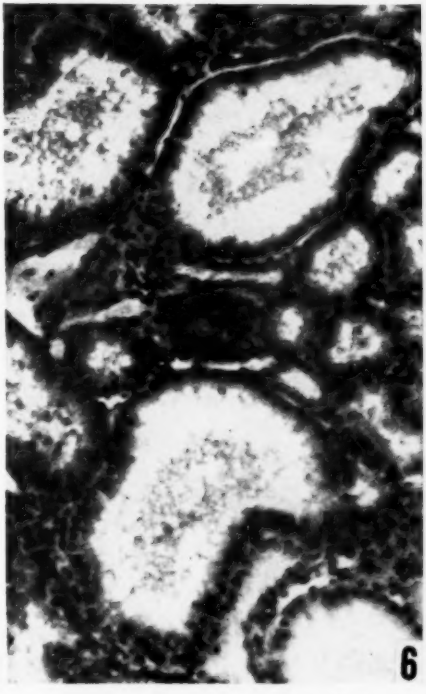

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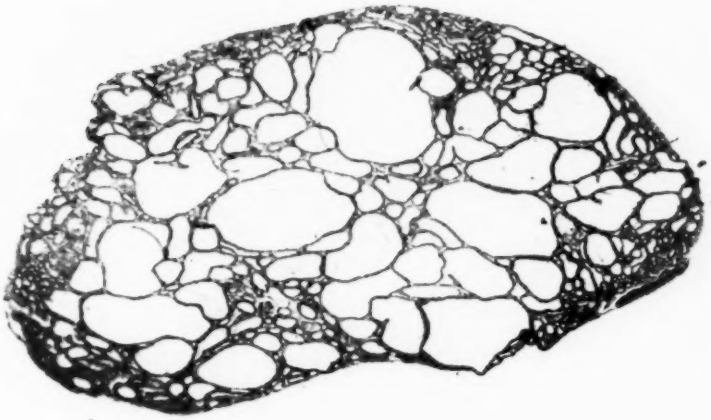
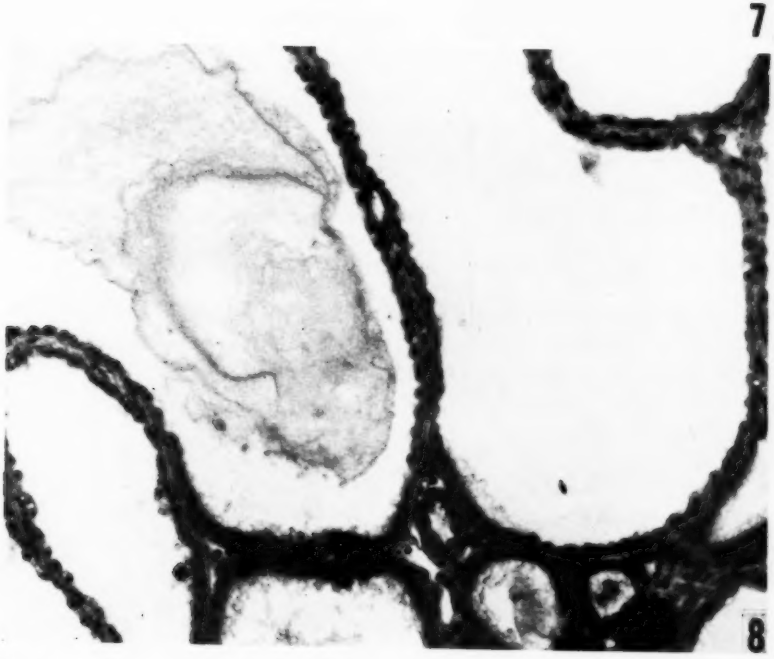
PLATE 74

FIGURE 7.—Cystic disease of the kidney of severe degree occurring in mice. × 12

FIGURE 8.—Cysts with flattened epithelium in severe polycystic disease of the kidney in mice. × 150





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Dose-Response Relations in Experimental Transmission of Avian Erythromyeloblastic Leukosis. V. Influence of Host Age and Route of Virus Inoculation[1,2]

EDWARD A. ECKERT,[3] DOROTHY BEARD, *and* J. W. BEARD, *Department of Surgery, Duke University School of Medicine, Durham, N. C.*

Procedures have been devised (*1*, *2*) and applied for measurement of host response to the virus of avian erythromyeloblastic leukosis (*1*, *3*). With these methods, which provide estimates of relatively high accuracy for an animal virus (*2*, *4*), it is possible to estimate the influence of factors which may alter either the infectious properties of the agent or the capacity of the host to respond. Experiments have already been reported (*5*) which reveal the character and extent of variations in response based on host-strain differences. It has been recognized that the age of the chick host at the time of exposure to the agent is an important factor in response and also that the response varies with the route of inoculation of the host with the agent. In view of the quantitative aspects of the methods now available, it has seemed desirable to re-examine the problem. In the present paper there are described the results of experiments on the bearing of age and route of inoculation of the virus on host response to the agent.

Materials and Methods

The disease investigated was the B. A. I. strain A of erythromyeloblastic leukosis originating as described in previous reports (*2*, *3*). The present work has been concerned with host response to the virus present in plasma from diseased chicks. The test plasmas were selected (*6*, *7*) for high content of virus, freed of cells by centrifugation and passed through celite and Selas filters (*8*).

The test hosts were White Leghorn chicks of Line 15 (*2*, *5*) developed at the Regional Poultry Research Laboratory at East Lansing, Michigan. In the experiments on the influence of age, injections of 0.1 ml.

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[2] This work was supported by a research grant to Duke University from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service; by a grant from the American Cancer Society, on recommendation of the Committee on Growth; by a grant from the Damon Runyon Memorial Fund for Cancer Research, Inc.; by the Dorothy Beard Research Fund; and sponsored by the Commission on Influenza, Armed Forces Epidemiological Board, and supported, in part, by the Office of The Surgeon General, Department of the Army.

[3] Scholar in Cancer Research of the American Cancer Society, Inc.

were made intravenously into chicks 3, 10, 17 and 24 days old. The older chicks had been kept from successive weekly hatches, and all were tested with the same virus preparation and on the same day. All of the chicks were derived from the same laying flock (*2*, *5*).

In studies on route of inoculation, the inoculums consisted in 0.1 ml. of plasma or dilutions prepared with BSA-Simms' solution (*1*) in the usual way, and the chicks were 3 days of age at the time of injection. Inoculations were made (*1*) into the wing vein; (*2*) into the medullary canal of the tibia; (*3*) into the muscle of the right leg; (*4*) subcutaneously over the right breast; (*5*) and into the peritoneum at the level of about 1 cm. to the right of the umbilicus. Injections into the medullary canal were made simply and rapidly with a #25-gauge needle with a very short bevel. The site was the posterior aspect of the lower tibia 2 to 3 mm. proximal to the epiphysis. Here the bone was soft, and puncture was effected by slight pressure associated, sometimes, with a slight twirling of the needle. Successful entrance into the canal was indicated by the feel of the needle dropping into the space and by the obstruction afforded by the opposite bone wall. Needles of the same kind were used for the intraperitoneal injections.

Analyses of host response were made with the latent-period procedures (*2*) developed for the virus of erythromyeloblastic leukosis.

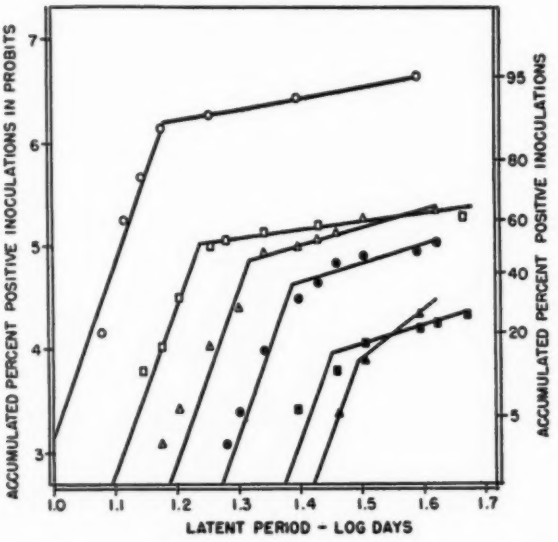
Results and Interpretations

The data of two experiments on the influence of age on the response of test chicks are given in table 1. In text-figure 1 there is shown graphically an example of the findings observed with the methods of analysis employed. These curves represent the time-frequency distributions of responses of six groups of the 3-day-old chicks of experiment 124 to inoculation with serial tenfold dilutions of plasma (see table 1). The distributions are qualitatively similar to those observed with all strains and lines of chicks thus far studied (*5*). It has been found that chicks of the Line 15 are of relatively high homogeneity and susceptibility in their response to the virus of erythromyeloblastic leukosis. The results of the present work were no exception. The lines through the points of the first limbs of the truncated curves were drawn with the standard slope [reciprocal of the standard deviation, 0.058 (*2*)] observed to be essentially constant for the chicks of this line. This indicates that the homogeneity of the population used in the present experiments was quantitatively similar to that of the hosts previously employed (*2*, *5*) with respect to the characters of the normal distribution of susceptibility or resistance. The curves were likewise similar to those reported earlier (*2*, *5*) in revealing the effects of resistance acquired by the host as the result of contact with the virus which causes truncation of the normal distribution after progressively longer periods of time after inoculation with diminishing doses of the agent.

TABLE 1.—*The responses of chicks of different ages to intravenous inoculation with the virus of erythromyeloblastic leukosis in two similar experiments*

Expt. No.	Age of chicks (*days*)	Log dose plasma (*ml.*)	Corrected No. chicks inoculated	Median log latent period (*days*)	Median latent period (*days*)	Log latent period at truncation (*days*)	Latent period at truncation (*days*)	Positive at truncation (*percent*)	Total positive (*percent*)
124	3	−1	40	1. 112	12. 9	1. 184	15. 3	88	95. 0
	10	−1	46	1. 181	15. 2	1. 200	15. 9	56	67. 4
	17	−1	48	1. 199	15. 8	1. 190	15. 5	39	50. 0
	24	−1	44	1. 256	18. 0	1. 190	15. 5	14	18. 2
	3	−2	36	1. 236	17. 2	1. 244	17. 5	52	61. 1
	10	−2	43	1. 268	18. 5	1. 252	17. 9	37	48. 8
	17	−2	44	1. 281	19. 1	1. 234	17. 1	16	34. 1
	24	−2	42	R*	R	R	R	R	2. 4
	3	−3	36	1. 328	21. 3	1. 330	21. 4	47	63. 9
	10	−3	38	1. 375	23. 7	1. 351	22. 4	31	44. 7
	17	−3	40	1. 410	25. 7	1. 327	21. 2	8	12. 5
	24	−3	43	R	R	R	R	R	0. 0
	3	−4	39	1. 410	25. 7	1. 392	24. 7	38	51. 3
	3	−5	35	1. 514	32. 7	1. 468	29. 4	15	25. 7
	3	−6	38	1. 564	36. 6	1. 507	32. 1	15	26. 3
127	3	−1	37	1. 002	10. 1	1. 062	11. 5	77	91. 9
	10	−1	40	1. 083	12. 1	1. 100	12. 6	55	77. 5
	17	−1	39	1. 099	12. 6	1. 081	12. 1	30	35. 9
	24	−1	49	1. 190	15. 5	1. 090	12. 3	6	10. 2
	3	−2	38	1. 124	13. 3	1. 142	13. 9	60	78. 9
	10	−2	38	1. 188	15. 4	1. 166	14. 7	38	55. 3
	17	−2	38	1. 240	17. 4	1. 200	15. 9	16	26. 3
	24	−2	49	R	R	R	R	R	4. 1
	3	−3	39	1. 233	17. 1	1. 245	17. 6	54	66. 7
	10	−3	39	1. 331	21. 4	1. 285	19. 3	18	30. 8
	17	−3	41	1. 345	22. 1	1. 260	18. 2	7	12. 2
	24	−3	49	R	R	R	R	R	0. 0
	3	−4	37	1. 308	20. 3	1. 280	19. 1	30	43. 2
	3	−5	39	1. 401	25. 2	1. 351	22. 4	30	41. 0

* R signifies abnormal patterns of response related to high levels of resistance.



TEXT-FIGURE 1.—Time-frequency distributions of host response of 3-day-old chicks to a series of six serial tenfold dilutions (see table 1) of plasma containing the virus of erythromyeloblastic leukosis in experiment 124.

A series of similar distributions was found with all age groups, as will be seen later. From these data there were calculated (*9*) the median log latent period in days of the responses in the first limb of the distribution for each dilution of virus in all age groups (table 1). The resulting individual points were plotted for experiments 124 and 127 in text-figure 2 in relation to dose. Lines were drawn through these points with the standard slope, 0.104, characteristic (*2*) of the relation between median log latent period and log dose for Line 15 chicks. The excellent fit of the points to the lines is evidence again that whereas, as will be seen, the levels of natural resistance changed with age, there was no alteration in the character of the distribution of this quality in the host population.

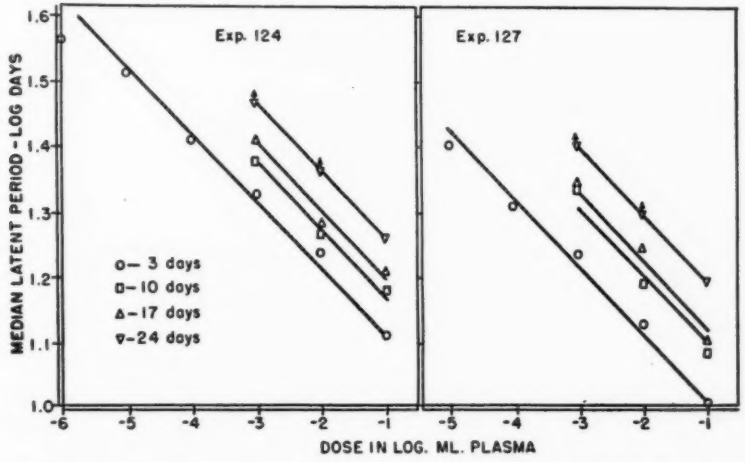
These results show that with increase in age there was progressive lengthening of the latent period indicative of increase in the levels of resistance of the respective groups. Employing the response of the 3-day-old chicks as the standard of reference, calculations were made, on the basis of the parallel dose-response relations, of the factors of increase in the levels of resistance of the different age-groups. The values, given in table 2, showed unexpectedly large and rapid increases in resistance, averaging in the two experiments, 39.2-fold during the period of 21 days. While the results showed some variation, it was apparent that the rate of increase was practically logarithmic during the time of study.

The foregoing analyses were made on the basis of latent-period relations and do not reflect fully the over-all picture of total group increase in resistance. Complementary information, though less susceptible to analy-

TABLE 2.—*Relative resistance of chicks of different ages to the virus of erythromyeloblastic leukosis given intravenously*

Age (*days*)	Weight of 20 chicks* (*lbs.*)	Relative resistance		
		Expt. 124	Expt. 127	Average†
3.....	1. 75	1. 0	1. 0	1. 0
10.....	3. 0	3. 4	8. 5	5. 4
17.....	4. 25	6. 8	13. 0	9. 0
24.....	8. 75	22. 7	66. 7	39. 2

*Chicks in experiment 124.
†Averaged on log relative potency level.



TEXT-FIGURE 2.—The relations of median log latent period in days to log dose in the four groups of chicks of different ages in two different experiments. The points with arrows directed upward were indeterminate because of the low response of the oldest hosts. The lines were drawn for the 24-day-old chicks in both experiments through the single point which could be determined.

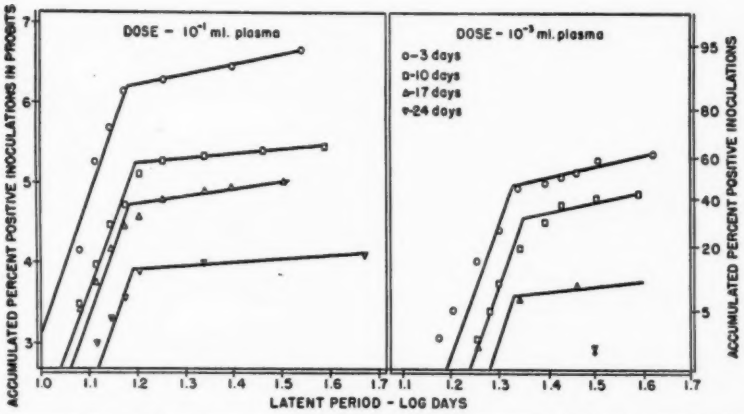
sis, is provided by reference to the record of positive inoculations resulting from the introduction of the same quantities of virus into the various age-groups. The values representing percent positive inoculations at the points of truncation and those of the total number of chicks positive are given in table 1. It is notable that only the values of percent positive at the point of truncation are significant for interpretation relative to increases in natural resistance with increasing age. These results obviously corroborate completely, in principle, the large changes measured accurately with the latent-period procedures.

A factor of possible importance in host-virus relationship was the variation in experimental conditions associated with natural increase in body weight and blood volume with age. In order to take into account this variable, weights were obtained on random samples of 20 chicks

of each age-group on the day of virus inoculation. The weights observed for each batch of chicks in experiment 124 are given in table 2, revealing a fivefold weight-increase in 21 days above the level at the 3-day age period. The dose-mass of virus was the same for all groups, but the immediate concentration of the agent in the circulation was progressively less with increase in the blood volumes. There is evidence (*10*) that the response to certain viruses is not governed wholly by the total mass of virus presented to susceptible tissues but is influenced somewhat by concentration of virus in the inoculum. In these experiments, as in studies (*11*) on the introduction of different amounts and volumes of influenza virus into the chorio-allantoic sac, there was no definite evidence that dilution was a significant factor in the apparent increase in resistance; on the contrary, as will be seen, the characters of the responses provided direct evidence that any possible influence of dilution was beyond the limits detectable by the method of analysis. It should be noted in both experiments that the rate of increase in resistance was greater in the first 7 days than in the interval of 10 to 17 days but larger again between 17 and 24 days. It is of interest that the rates of increase in body weight varied in the same way.

Information about the nature of the participation of the individual chick to over-all group response is to be derived by consideration of the time-frequency distribution curves of all groups of chicks inoculated with the same amounts of virus. Examples of these are shown in text-figure 3 which summarizes the results with the doses of 10^{-1} and 10^{-3} ml. of plasma in experiment 124. With the former dose it is seen that the slopes of the initial limbs of the truncated curves did not vary significantly with the different age-groups, and the lines were drawn, as in text-figure 1, with the standard slope (reciprocal of the standard deviation, 0.058), characteristic for Line 15 chicks. It is evident, therefore, that the characters of the normal distribution of resistance or susceptibility did not change with age; instead, statistically, the resistance of the various individuals increased proportionally, retaining the initial homogeneity of the group within the limits of the expression of natural resistance. There is illustrated graphically also the phenomenon of progressive decrease in the frequency of positive inoculations at the point of truncation as mentioned before.

A result of considerable interest is the fact that truncation of the various curves occurred at essentially identical periods after inoculation of the virus. This is clearly revealed in table 1 in the closely similar values of the latent period at truncation with each dose. Truncation, therefore, was related neither to the age of the bird nor to the latent period of infection in those individuals responding by development of the disease. This finding indicates that the process of the induction of acquired immunity is related only to time and to the dose of the agent administered and is independent, in those birds of different ages, of both the development of the infectious process and the level of increased natural resistance in the individual host. It is not meant to imply that the virus concerned



TEXT-FIGURE 3.—Time-frequency distributions of the response of the chicks of different ages to two different doses of plasma containing the virus of erythromyeloblastosis.

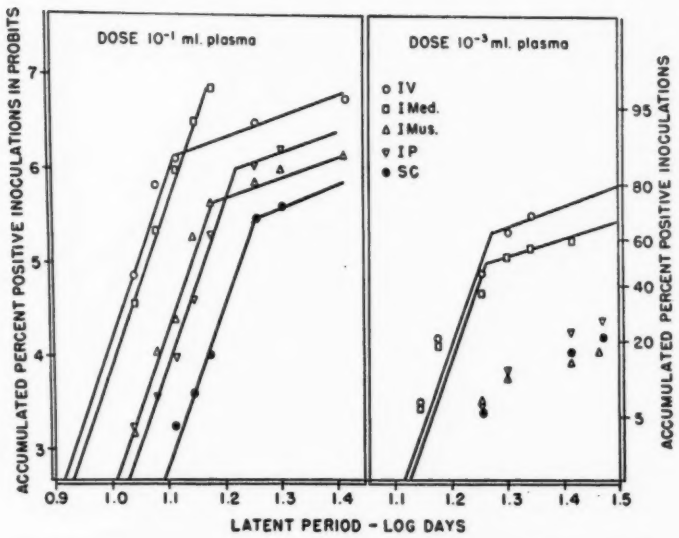
with and originating in the infectious process does not contribute to acquired resistance; statistically, however, the only relation detectable is that to the initial dose. The results with the smaller dose, 10^{-3} ml. of plasma, were qualitatively similar to those with 10^{-1} ml. plasma except that the period of time needed for the development of acquired immunity after inoculation was greater than that associated with the larger dose. The fact that truncation occurred at the same interval after inoculation of a given dose constitutes evidence that variable dilution of the inoculum by different blood volumes is not an important aspect of the manifestation of apparent increased resistance with age; variation in the inoculums at a given age always results in variation of the points of truncation (see text-fig. 1).

Studies on the influence of the route of virus inoculation were made in three experiments, 122, 123 and 125, as recorded in table 3. The results of experiment 125 are shown graphically, text-figure 4, in the form of the time-frequency distribution curves derived by inoculation of 2 different doses, 10^{-1} and 10^{-3} ml. of plasma, in 3-day-old chicks. The number of doses, table 3, which could be used was limited by the number of chicks available; consequently complete analyses could not be made on the basis of the parallel dose-response relations employed in the age experiments. It is clear, however, that the route of inoculation strongly affects the response of the host with respect to the latent period of infection. Again, as before, the time-frequency distributions were truncated; the individual points obtained in the first limbs of the truncated curves by inoculation with 10^{-1} ml. plasma fitted closely the lines of slope (reciprocal of the standard deviation, 0.058) standard for Line 15 chicks. This indicated, as before, that the characters of the distributions of resistance or susceptibility in the host groups were the same, independent of the manner of virus inoculation. It is observed, however, that the points of truncation vary (latent period at truncation, table 3) chronologically with the route

TABLE 3.—*The response of 3-day-old chicks to the virus of erythromyeloblastic leukosis administered by different routes*

Expt. No.	Route of inoculation*	Log dose plasma (*ml.*)	Corrected No. chicks inoculated	Median log latent period (*days*)	Median latent period (*days*)	Log latent period at truncation (*days*)	Latent period at truncation (*days*)	Positive at truncation (*percent*)	Total positive (*percent*)
122	IV	−1	37	1.035	10.8				100
	IMed	−1	37	1.075	11.9	1.110	12.9	69	78.4
	IP	−1	32	1.175	15.0	1.162	14.5	41	59.4
	SC	−1	38	1.232	17.1	1.245	17.6	52	65.8
	IV	−3	35	1.258	18.1	1.297	19.8	75	77.1
	IMed	−3	36	1.277	18.9	1.315	20.7	74	77.8
	IP	−3	34	1.432	27.0	1.381	24.0	21	20.6
	SC	−3	39	1.459	28.8	1.395	24.8	17	23.1
123	IV	−1	36	1.129	13.5	1.192	15.6	84	88.9
	IMus	−1	29	1.278	19.0	1.275	18.8	45	55.2
125	IV	−1	29	1.046	11.1	1.114	13.0	88	96.6
	IMed	−1	30	1.061	11.5			97	96.7
	IMus	−1	30	1.143	13.9	1.183	15.2	76	86.7
	IP	−1	26	1.164	14.6	1.216	16.4	84	88.5
	SC	−1	25	1.227	16.9	1.252	17.9	68	76.0
	IV	−3	29	1.251	17.8	1.270	18.6	63	69.0
	IMed	−3	27	1.260	18.2	1.257	18.1	48	59.3
	IMus	−3	29	R†	R	R	R	R	20.7
	IP	−3	26	R	R	R	R	R	26.9
	SC	−3	29	R	R	R	R	R	27.6

*IV=intravenous; IMed=medullary canal of tibia; IP=peritoneum; SC=subcutaneous tissue; and IMus=muscle.
†R signifies abnormal patterns of response related to high levels of resistance.



TEXT-FIGURE 4.—Time-frequency distributions of the response of 3-day-old chicks to two different doses of virus administered by different routes of inoculation. The responses to inoculums of 10^{-3} ml. of plasma given into muscle, peritoneal cavity and subcutaneous tissue were too low for normal analysis.

of inoculation and this in the way expected to follow inoculation of different amounts of virus by a given route in chicks of the same age.

Numerical expressions of the differences in response calculated from the median log latent-period relations with the available data are given in table 4. From the averages in all experiments, it is seen that the responses fall approximately into 3 groups. The response to injection of virus into the medullary canal of the tibia was similar, though significantly less than that to intravenous inoculation. The responses to administration of the agent intraperitoneally and into muscle were of another order and closely similar one to the other. Subcutaneous inoculation resulted in still considerably smaller response. The findings analyzed with the latent-period methods were corroborated in nearly all instances by the percent-

TABLE 4.—*Relative resistance of 3-day-old chicks to infection with the virus of erythromyeloblastic leukosis given by different routes*

Route of inoculation	Relative resistance			
	Expt. 122	Expt. 123	Expt. 125	Average*
Intravenous	1. 0	1. 0	1. 0	1. 0
Intramedullary	2. 4		1. 4	1. 8
Intramuscular		27. 0	8. 5	15. 2
Intraperitoneal	23. 3		13. 7	17. 5
Subcutaneous	76. 9		55. 5	66. 6

*Averaged on log relative potency level.

positive inoculations both at the points of truncation and total percent positive within the period of the experiments.

Discussion

The significance of the present findings resides, in part, in the demonstration of the quantitative aspects of host-virus relationships generally recognized to exist but not previously subjected to measurement in erythromyeloblastic leukosis. Of probably greater interest, however, is the new information which serves to clarify the characters of individual host response contributing to group behavior.

There is indicated in table 2 a very large dependence on age of chick test-group response to the virus of erythromyeloblastic leukosis. In two experiments, there was an average of about fortyfold increase in resistance in an age-period of only 21 days. This result had no bearing on the state of the agent or differences in the number of infectious virus particles since the inoculums were from the same source in all cases. It would appear that the progression to the higher levels of resistance was essentially logarithmic within the period of study, and it was clear that the rate of increase in natural resistance was far greater than that in weight. The processes underlying the increased capacity of the host to resist infection with the virus are obscure; the results suggest that factors related to the conditions governing growth might have been of influence on the change in the state of susceptibility or resistance since the rates of gain in resistance were greatest in the periods of most rapid growth. The course of the increase in resistance in the process of further aging is unpredictable, but it would seem unlikely that the rate would continue at the high level exhibited by the young birds. Studies with still older chicks were avoided because of difficulties of collecting adequate data due to high resistance and of frequent handling and maintenance of large chickens. It should be emphasized that the quantitative aspects of the results both of the studies on age influence and the effects of different routes of inoculation are not necessarily applicable to all strains and lines of chicks. It is probable that all chicks would behave in a similar manner but in variable degree.

The methods of analysis employed were highly effective not only in the quantitative estimation of changes in average or group response related to age but in the clarification of questions of the contribution of individual chick factors. Each chick begins with its own level of susceptibility and with growth and aging, the bird becomes increasingly refractory to the infectious process. It is apparent that this increase, statistically, is not proportional to the initial state of natural resistance but is essentially the same in all of the birds. Had this not been the case, there would have occurred a change in the slope of the initial limb of the time-frequency curve. With time, presumably in relation to growth and the maturing process of the chick, resistance acquired by virtue of the immunological experience with the virus becomes added to the state of natural resistance, affecting the response of every bird which had not developed infection by

the end of the critical period. In these experiments, this period between inoculation and manifestation of the interference by acquired resistance was the same at all ages with a given dose, as indicated by a similar latent period at truncation. It is thus evident that at some time within the latent period there was rather suddenly added to the natural resistance of the individual birds an amount of acquired resistance. Beyond this point the analyses do not permit interpretation of quantitative differences with respect to the pattern of the addition, that is, whether the character and level of the natural resistance of the chick is a measure of or a factor in the rate of development or level of acquired resistance. There is no doubt that the pattern of total natural and acquired resistance, though perhaps still normally distributed, is greatly different from that of the natural state before the impression of the influence of acquired resistance.

It should be emphasized here that the present findings add materially to the knowledge of immunological processes in this disease. There seems no question that the response recognized after truncation is related to the development of a state of relative active immunity to the virus of erythromyeloblastic leukosis. There has been much variation in earlier findings (*12*) in attempts to detect and measure the effects of vaccination with various virus preparations on the resistance of treated birds. Though demonstration of immune bodies has been reported in studies on leukosis, it has been the experience in this laboratory that such bodies, if they exist, are distinctly meager and very unlikely to account even in a small way for the profound changes indicated in the levels of resistance beyond the truncation of the time-frequency curves. The data do indicate unequivocally, however, a large influence of the presence of the virus in the host on the immunological processes which determine the total level of resistance. In brief, birds injected with the virus develop an active immunity to the infectious process, and this is related to the amount of virus introduced and, as observed, to the route of inoculation. The results serve to stress certain of the difficulties expected in any investigation on the development of acquired active immunity. Change in natural resistance and natural high resistance, together with other variable factors, would add greatly to the obstacles to quantitative measurement of the effects of vaccination or exposure of the host to virus material.

The studies on the influence of route of inoculation of the virus showed no deviation in the characters of the disease related to the method of administration. It was apparent that the pattern of response was normal throughout for Line 15 chicks, although profound differences were seen in the levels of resistance or susceptibility in the various test groups as measured by the latent-period method. As noted before, the series of curves resembles one which might have resulted from the inoculation of different amounts of virus intravenously. For example, it would appear, for all practical purposes, that only 100/66.6 (average in experiments 122 and 125, table 4) percent of the virus inoculated subcutaneously ever became available at the site of susceptible tissue for the induction of infection. Variations in truncation of the time-frequency curves indicated

also that virus was lost not only as the agent responsible for infection but as antigen responsible for stimulation of the immune process.

Summary

Studies were made on the influence of host age and route of inoculation on the response of chicks to the virus of avian erythromyeloblastic leukosis. In two experiments there were group increases in natural resistance in 21 days to levels 22.7- and 66.7-fold the level present at the 3-day period of age. The data not only revealed the characters of group response but provided information interpretable with respect to the contribution of the individual host. There were revealed, also, evidence of acquired active resistance and the effects of acquired resistance on individual and group response. Measurements were made of the levels of host response to virus inoculated intravenously; into the medullary canal of the tibia; into muscle; into the peritoneal cavity; and subcutaneously. The results showed large differences, and the characters of the responses were similar to those which might be expected following the inoculation of different amounts of virus by one route of administration.

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Virus of Avian Erythromyeloblastic Leukosis. V. *p*H Stability of the Virus Particles, the Infectivity and the Enzyme Dephosphorylating Adenosine Triphosphate[1,2]

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Plasmas of chicks with erythromyeloblastic leukosis (*1*) contain a particulate material (*2*, *3*), the virus entity, and an enzyme (*4*) dephosphorylating adenosine triphosphate. The three attributes occur proportionately in the plasmas (*5–7*) and are inseparable by sedimentation or electrophoresis. It has been judged that the particles represent the virus which possesses the specific enzyme activity. In establishing the basic conditions for study of the etiologic agent of the disease, investigation has been made to learn the *p*H limits favorable for stability of the infectious quality of the virus. Concurrently, there were determined, on the same materials, the effects of the various hydrogen-ion concentrations on the enzyme activity and on the integrity or characters of the particles studied and counted by electron microscopy. The results of this work are described in the present paper.

Materials and Methods

The disease was the B. A. I. strain A of erythromyeloblastic leukosis isolated by Hall, Bean and Pollard (*8*). This was the strain obtained from Dr. E. P. Johnson and employed in previous work from this laboratory.

Blood providing the plasma was obtained and the cells were removed by centrifugation as already described (*5*). The chick donors were White Leghorns of Line 15 (*9*) (Regional Poultry Research Laboratory, East Lansing, Mich.) inoculated with previous passage virus at the age of 3 days. Prospective donors were selected with the micro-screening method (*10*, *11*) in an advanced stage of the disease as indicated by high concentrations of primitive cells seen in routine blood smears (*9*). Several chicks, 4 to 7, were bled to provide material for each experiment, and the

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[3] Scholar in Cancer Research of the American Cancer Society, Inc

time of bleeding was 3 to 6 weeks after inoculation. The plasmas for a given experiment were pooled after removal of the cells, diluted with an equal volume of Ca-free Ringer's solution, filtered first with celite #512, and then passed through 2-inch Selas filter candles of porosity 02 (FP-52, Scientific Equipment Division, Selas Corporation of America, Philadelphia, Pa.). All operations were carried out in the cold.

A series of 3 experiments, experiments 103, 104 and 107, was made, differing in the manner of attaining the desired *p*H and in the period of exposure of the virus to the various levels of hydrogen-ion concentration. In all cases the virus was concentrated and partly purified by a single sedimentation in the ultracentrifuge. The plasma of experiment 103 was freed of cells and stored in the refrigerator overnight prior to filtration and ultracentrifugation for the experiment made on the following day. In the other two instances, filtration and virus concentration were carried out on the day of bleeding, and the *p*H mixtures were set up immediately afterward. In order to concentrate the virus the plasma was spun in 15-ml. celluloid tubes for 45 minutes at 20,000 $\times$ g. The supernatant plasma was poured off, the walls of the tubes were wiped with a gauze sponge, and the pellets in a given experiment were resuspended in 2 ml. of Ca-free Ringer's solution. Each suspension was centrifuged for 10 minutes at 3,000 r.p.m. in 5-ml. celluloid tubes in the angle head of the International centrifuge #2. The supernatant, opalescent suspension, which contained the virus, was pipetted from the scarcely visible sediment.

In each experiment, the concentrated virus suspension was diluted to 8 ml. with Ca-free Ringer's solution, parceled in 1-ml. volumes, and *p*H adjustment was made with 6 specimens. In experiment 103, the *p*H was brought to the desired level, as determined with the glass electrode, by the addition to the 1-ml. volumes of sufficient HCl or NaOH in 0.1 to 0.01 *N* concentration. After a period of 10 minutes, the *p*H was quickly readjusted to 7.0 with acid or base. All procedures were carried out at 26° C. The desired *p*H levels were attained in experiments 104 and 107 with composite buffer containing phosphate, borate and phthalate. Stock solutions were prepared to contain K_2HPO_4, H_3BO_3 and the monopotassium phthalate. The *p*H was adjusted with acid or alkali, and the respective mixtures were diluted to contain the buffer salts each in 0.05 *M* concentration. For each test mixture there was added 0.3 ml. of the required buffer solution to the 1.0-ml. volume of virus suspension. The final concentrations of each buffer was 0.0115 *M*, and the total concentration of buffer salts was 0.0345 *M*. Readjustment of *p*H in experiment 104 was made after 18 hours and in experiment 107 after 63 hours. The mixtures were kept at 4° C. during these periods. The *p*H was adjusted approximately to 7 by the addition to each preparation of 0.7 ml. of 0.02 *M* phosphate buffer of *p*H 7.0. The total volume of each mixture was then made to 2.0 ml. with Ca-free Ringer's solution. The preparations were sampled at this point for the minute volumes needed for particle counts and measurement of enzyme activity. The time sequence of the period of exposure and the subsequent manipulations

was such that the final adjustment of *p*H was made only a few minutes before the materials were inoculated for infectivity measurements.

Infectivity measurements (*9*) were made in White Leghorn chickens of the Line 15 mentioned in a previous paragraph. A volume of 1 ml. of each test virus mixture was diluted to 6.0 ml. with bovine serum albumin in Simms' solution (*12*) of *p*H 7.2 to 7.4. This suspension was injected in 0.1-ml. volumes intravenously in 35 to 40 three-day-old chicks. Blood smears were made as usual to determine the latent period of infection induced by the inoculums. In all experiments, a second inoculum was prepared of the test suspension of *p*H 7. This was a 100-fold dilution of the suspension, and it was injected as were the other inoculums. Analyses of the data were made as described (*9*) in the method of titrating the virus. The levels of infectivity are stated here as the median log latent periods found with each material inoculated from the 6.0-ml. volumes; the results obtained with the 10^{-2} dilution of the *p*H 7 material were used only as a check of the slope of the regression of latent period on dose (*9*).

The procedure for counting the virus particles was the same as that already described (*5*), except that each agar surface was stripped twice [4] with collodion films. Estimations of the activity of the preparations to dephosphorylate adenosine triphosphate were made electrotitrimetrically (*5*), using reaction mixtures containing balanced saline solution (*11*).

Results

The data of the three experiments are given in table 1 and summarized graphically in text-figures 1*A* and 1*B*. In text-figure 1*A*, the levels of infectivity are shown on a relative basis employing, in each experiment, the value obtained with the preparation held at *p*H 7 as 100 percent active. This provides no information about absolute decreases in activity in all regions of the *p*H range associated with the various time intervals. Nevertheless, stability relative to *p*H and the time factors and the regions optimum for stability are clearly evident with this method of presentation. Calculations of changes in enzyme activity, text-figure 1*B*, were made in the same way. Use was made of log-percent change in order to bring the results of infectivity measurement and enzyme activity together on the same coordinates.

In the region of *p*H 5.3 to 9.1 the properties of the virus relative to infectivity were fully stable for 10 minutes. Infectivity was destroyed at *p*H 2.5, and there occurred a very appreciable inactivation at *p*H 10.4 in the same period. After exposure of the virus to the various hydrogen-ion levels for 18 hours, infectivity of the preparations at about *p*H 6 and *p*H 9 was considerably less, about 60 to 70 percent, than that at *p*H 7. Similar results were seen after the 63-hour interval at *p*H 9; the point at *p*H 5.7 in the same experiment exhibited a higher level of infectivity

[4] Recent observations have shown that pseudoreplicas from agar sometimes fail to remove all the virus sedimented from plasma. Second replicas have been found to remove practically all the virus so that the counts reported here are the sum of first and second replicas.

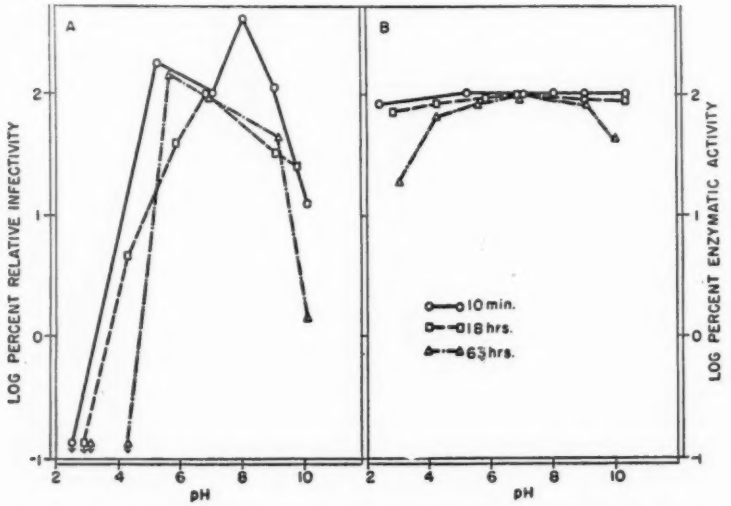
TABLE 1.—*pH stability of the infectivity, enzyme activity and particle integrity of the virus of avian erythromyeloblastic leukosis*

Expt. No.	pH	Median log latent period*	Phosphorus liberated†	Particle number‡
		(*days*)	(*gamma*)	(× 10^9)
103	2. 5	1. 641	138. 9	343
(10 Min.)	5. 3	1. 248	168. 6	157
	7. 1	1. 272	168. 6	373
	8. 1	1. 196	168. 6	153
	9. 1	1. 266	168. 6	267
	10. 4	1. 364	168. 6	327
104	2. 9	1. 6	143. 8	251
(18 Hr.)	4. 3	1. 287	158. 7	226
	5. 9	1. 195	188. 5	230
	7. 0	1. 153	203. 4	220
	9. 1	1. 201	188. 5	192
	9. 8	1. 213	183. 5	224
107	3. 1	1. 6	69. 4	388
(63 Hr.)	4. 3	1. 446	233. 1	299
	5. 7	1. 110	307. 5	356
	7. 0	1. 126	372. 0	395
	9. 2	1. 157	327. 4	402
	10. 1	1. 311	168. 6	366

*Median log latent period of infection induced in test-chick groups by 1.6×10^{-3} ml. reaction mixture injected into each chick.

†*Gamma* phosphorus liberated per minute by 1 ml. of the reaction mixture.

‡The number of particles per ml. of the reaction mixture.



TEXT-FIGURE 1.—*A.* Effect of hydrogen-ion concentration on infectivity of the erythromyeloblastic leukosis virus during intervals of exposure of 10 minutes, 18 hours and 63 hours. The *arrows* indicate values less than 0.1 percent residual infectivity.

B. Effect of hydrogen-ion concentration on the enzyme activity to dephosphorylate adenosine triphosphate of the same preparations as those of *A*.

than that of the preparations at *p*H 7. Some of the apparent differences in the stability of the various preparations are doubtless related to variations inherent in the factors determining the accuracy of the titration method. It may be seen, however, that stability is probably at the maximum near *p*H 7, and that outside the limits of the range of *p*H 6 to *p*H 9, the virus is quickly inactivated, more rapidly so on the acid side than in the alkaline region.

The results portrayed in text-figure 1*B* show that the material responsible for enzyme activity is more stable than that associated with infectivity. An effect within 10 minutes was seen only at the extreme of *p*H 2.5. More influence was evident in 18 hours. The curve at 63 hours shows that the optimum *p*H for stability is near 7. As in the case of infectivity, the adverse influence of hydrogen-ion concentration was greatest in the acid region and the range of lesser effects on the enzyme was the same as that seen with infectivity, namely, between a *p*H of about 6 to *p*H 9.

There was no indication in any of the 3 experiments of any effect on the physical character of the particulate component which would change its appearance or the number of particles seen in electron micrographs. Two low counts were obtained in experiment 103, but these were not in the extreme *p*H regions where the greatest changes occurred in infectivity and enzyme activity.

Discussion

The results of the present work indicate a relatively narrow *p*H range favorable for stability of the infectious capacity of the erythromyeloblastic leukosis virus. On the average, infectivity remained highest at a *p*H of about 7, and the activity was lost relatively slowly in the region of *p*H 6 to *p*H 9. This behavior is not greatly different from that of most other animal viruses. It is of some interest that the region of optimum stability of the papilloma virus, another agent associated with tumor induction, extends (*13*) well into the acid region, whereas the erythromyeloblastic leukosis agent differs in its susceptibility to dilute acid.

It is notable that the range optimum for stability of the enzymatic activity to dephosphorylate adenosine triphosphate was essentially the same as that for the infectious quality of the virus particle. The enzyme activity, which could be measured much more accurately than infectivity, appeared somewhat more stable than the latter, but loss of enzyme effect was considerable at *p*H levels below 7 and above 9. These similarities of the respective regions of *p*H stability may be of some significance in relation to earlier findings correlating virus infectivity, and enzyme activity with the particulate component. There has been no reason, however, to suppose that the property of infectivity and that of enzyme activity are associated with the same constituent of the particle and, consequently, that conditions adverse to one quality would directly affect the other in the same manner. That physical changes in the virus particle were not drastic under any conditions of the experiments was indicated by the

characters and numbers of the particles in electron micrographs. The lack of change in the particles despite loss of infectivity is a finding in common with the results of studies on other viruses which have shown the loss of infectivity without degradation of virus particles detectable in sedimentation diagrams.

Summary

The infectious quality of the erythromyeloblastic leukosis virus is most stable at *p*H about 7, and the region favorable for relative stability extends from *p*H about 6 to 9. Enzymatic activity of the same preparations to dephosphorylate adenosine triphosphate was more stable than infectious capacity, but the *p*H regions of optimum stability were essentially identical. There was no evidence in electron micrographs of effect on the physical characters of the particulate material of the plasma nor were there any differences in the numbers of the particles related to hydrogen-ion concentration.

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Virus of Avian Erythromyeloblastic Leukosis. VI. Properties of the Enzyme Associated with the Virus in Dephosphorylating Adenosine Triphosphate[1,2]

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The plasma from the blood of chicks with erythromyeloblastic leukosis (*1*) exerts an enzyme activity (*2*) to dephosphorylate adenosine triphosphate. In the same plasmas there occur, also, a specific particulate component (*3, 4*) and the etiologic virus responsible for the disease. It has been shown (*5–8*) that enzyme activity, particles and infectious capacity appear simultaneously and proportionately in the plasma with development of this form of leukosis, and that the three attributes exhibit essentially identical electrophoretic mobilities and sedimentation properties. There is, thus far, no evidence contrary to the view that the particles represent the virus and that the virus carries with it the specific enzymatic activity. Should the phenomenon prove, finally, to be related to an intrinsic constituent of the virus, its significance is of more than ordinary interest. There is a wealth of fundamental implications with respect to possible contributions of such a factor to the physiologic or reproductive processes of the agent *per se* and to the processes associated with those influences of the agent on the host cell which result in transformation of the cell from the normal to the malignant state. In addition, the activity is of much practical importance in providing valuable relations and procedures (*9, 10*) for expediting study of the agent in the selection of chick donors as source of the virus and in the elimination of the necessity for innumerable biological assays based on virus infectivity. Prosecution of the studies already reported (*2, 4–10*) has been based on the results of preliminary investigations of the properties of the enzyme, some of which have been noted from time to time. The procedures employed in the work and the findings encountered are reported in detail in the present paper.

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[2] This work was supported by a research grant to Duke University from the American Cancer Society, on recommendation of the Committee on Growth; by a grant from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service; by the Dorothy Beard Research Fund; it was also sponsored by the Commission on Influenza, Armed Forces Epidemiological Board, and supported, in part, by the Office of The Surgeon General, Department of the Army.

Materials and Methods

Blood plasma.—The disease under study was the virus-induced form of the avian leukosis complex commonly designated as erythromyeloblastic leukosis (*1*). Pathologic studies, at one period since work was undertaken with this disease, have indicated (*11*) that the circulating primitive cells characterizing the condition were myeloblasts, and no evidence was seen of erythroblasts. Inasmuch, however, as the interrelations between erythroblastic and myeloblastic leukosis are uncertain, the more general terminology has been retained. As a matter of routine, the disease is passed by means of the virus in frequent titration experiments. In all of the work reported here the passage was accomplished by intravenous injection (*12, 13*) of 0.1-ml. inoculums of plasma or partly purified virus into 3-day-old White Leghorn chicks of the inbred Line 15 originating (*13, 14*) at the Regional Poultry Research Laboratory at East Lansing, Michigan. When the chicks were in an advanced stage of the disease, as indicated by the large number of primitive cells seen in blood smears taken at frequent intervals (*13*), individuals with plasma of high enzyme and virus content were selected by the micro-screening test (*9, 10*) for estimating activity to dephosphorylate adenosine triphosphate. Blood was collected in chilled tubes by heart puncture or by section of the saggital sinus, with heparin as anticoagulant. The cells were removed by centrifugation as already described in detail (*5*), and the plasma was passed through celite and Selas filters (*5*). During all of these procedures the blood and plasma were kept in tubes either immersed in ice water or stored in the refrigerator.

Measurement of enzymatic activity.—Estimation of enzymatic activity was made by electrometric titration with essentially the apparatus described by Borsook and Dubnoff (*15*) with minor modifications. The reaction vessel, a 50-ml. beaker, was kept immersed above the level of the reaction mixture in a water bath at 30° C. and was provided with a close-fitting lid machined from lucite in which holes of the proper size were bored for passage of the pair of electrodes, the stirrer, and the pipette for adding KOH to the solution. The space about the electrodes was sealed with Plastilina, a modeling clay, and a small rubber disc made an effective seal about the opening for the pipette. An atmosphere of nitrogen[3] above the reaction mixture was attained by constant flow of the gas through a tube passing through another hole in the lucite lid. A hole was bored also to allow introduction of the reaction materials. After the reaction, the mixture and wash-fluids were removed by suction through the hole made for the KOH-pipette without the need for disassembly of the apparatus.

The procedures for measuring enzyme activity under conditions now regarded as optimum have evolved from the results of experiments such as those described in the present paper and are as follows:

The substrate for the reaction is adenosine triphosphate, either the disodium or the dipotassium salt (Pabst Laboratories). This is dissolved

[3] Use of the nitrogen atmosphere was necessary only in those experiments in which estimations were made with the null point in the reaction vessel above *p*H 7.5.

in an amount of 300 mg. in a final volume of 50 ml. of a saline solution containing, KCl, 0.05 *M;* NaCl, 0.05 *M;* $CaCl_2$, 0.04 *M;* and $MgCl_2$, 0.04 *M*. The *p*H is adjusted to 7.0 with 0.1 *M* KOH before the volume is made to 50 ml. The precipitate which forms in the solution apparently does not interfere with the subsequent reaction, but precautions are taken to keep the material dispersed during sampling. The solution is parceled in volumes of 5 to 6 ml. and stored at −18° C. Tests have been made from time to time on such fresh stock solutions to determine the concentration of adenosine triphosphate. Total nucleotide concentration was estimated with the Beckman spectrophotometer at a wave length of 2,600 Å after determining this as the maximum in the absorption spectrum. Calculation was made (*16*) of nucleotide concentration from the molar extinction coefficient of 16,000 at *p*H 7. Possible degradation of the adenosine triphosphate to adenosine diphosphate was investigated by determination of free inorganic phosphate with the method of Fiske and Subbarow (*17*). Tests for the presence of adenylic acid-5 were made by passage of an aliquot of the solution over Dowex-1 (chloride cycle) contained in a $cm.^2$ by 4 cm. glass column fitted with a fritted glass tip (*18*). The eluates from the resin were then tested for adenylic acid, adenosine triphosphate and adenosine diphosphate with the Beckman spectrophotometer (*16*). The adenosine triphosphate salts employed were never less than 95 percent pure, the residual material consisting essentially of adenosine diphosphate which, in the small amounts found, was not sufficient to cause significant inhibition in the initial rate of the reaction.

Estimations of enzyme activity are made in final volumes of 25 ml. This is constituted of the saline solution described above (previously brought to 30° C. by immersion in the constant temperature bath) in a volume which, together with the volume of plasma or concentrated virus, totals 24 ml. The *p*H of the mixture is adjusted with 0.01 *M* KOH solution to 7.0 and the reaction is initiated by the addition of 1.0 ml. of the cold adenosine triphosphate solution. Shifts from the null point of *p*H 7.0 are negligible. A small volume, 0.01 to 0.02 ml., of the KOH solution is quickly added to bring the reaction very slightly to the alkaline side of the null point, and zero time for the reaction is established at the moment the needle of the Beckman meter returns to *p*H 7.0. The rate of reaction is then followed by notation of the time for the *p*H to return to the null point on repeated additions of small volumes of standardized 0.01 *M* KOH. The reaction rate, determined in terms of microequivalents of acid produced as a function of time, is converted to micromoles of phosphate, using the factors relating phosphate to acid liberated at *p*H 7.0 in the presence of the ions employed for the reaction ATP→ADP + P as was determined in the present experiments.

Experiments and Results

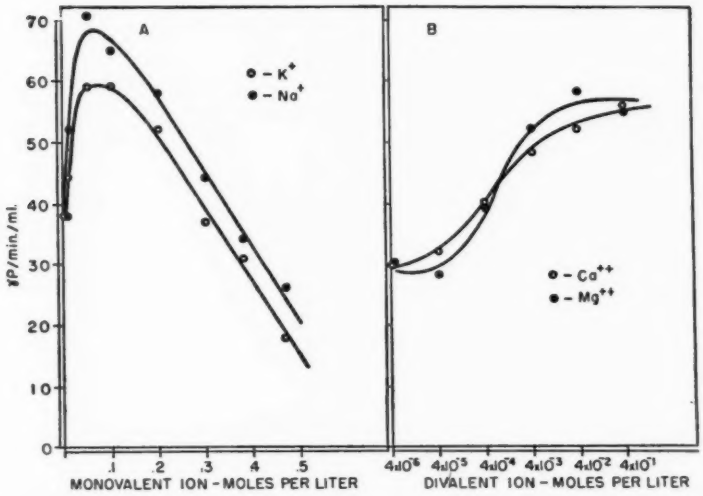
Normal plasma controls.—During the course of the study of the enzyme dephosphorylating adenosine triphosphate since January 1952, numerous examinations have been made to test the activity of the plasmas from

normal chicks. For the present purpose, "normal" has been used to indicate birds which had not been injected with the virus of avian erythromyeloblastic leukosis and which showed no evidence, by blood smear, of the presence of the disease. No enzymatic activity has been detected in any case with such plasmas whether examined by direct phosphorus determinations (*17*), by electrometric titration or by the micro-screening test (*9*, *10*). In order to provide specific data for this report, tests were made with the microdephosphorylation reaction (*10*) on the same day on a total of 56 birds, 10 of which were 19 weeks; 7 of 18 weeks; 7 of 15 weeks; and 32 of 13 weeks of age. These chicks were White Leghorns of the same Line 15 (*14*) routinely employed for other studies of enzyme activity and had not been injected with the virus. They had been kept in the same room, though in different cages, housing several hundred chicks injected and diseased with the virus. In these tests there was no significant color change in the indicator solution at room temperature in the period of one hour. It is thus evident that the plasmas from these normal chicks did not contain the enzyme in significant amounts (*10*).

Ion effects on enzyme activity.—Investigations were made to determine possible effects of the principal cations expected to be present in the plasma of the affected birds, normally, Na^+ and Ca^{++}. It was suspected that K^+ and Mg^{++}, also, might appear at elevated levels in the plasma of diseased birds in association with possible breakdown of circulating cells. A subsidiary investigation in this respect revealed, as described in another report (*19*), that both ions reach high concentrations in the diseased plasmas, showing a close correlation with the enzyme activity, and, consequently, with the number of virus particles of the plasmas.

The effects of Na^+, K^+, Ca^{++} and Mg^{++} acting separately in dephosphorylation by the plasma are shown in text-figure 1*A* and 1*B*. For these experiments, a solution of adenosine triphosphate was prepared by dissolving 300 mg. of the salt in distilled water in a final volume of 50 ml., with adjustment of the *p*H to a level above 7.0 such that addition of 1.0 ml. of the solution to the reaction mixture did not effect an initial quick shift in the *p*H from 7.0. The volume of plasma employed was 0.5 ml., and the respective reaction media consisted of 23.5 ml. of various concentrations of NaCl, KCl, $CaCl_2$, or $MgCl_2$. These, as well as all other studies, were made at 30° C.

The results obtained in an experiment with Na^+ and another with K^+ are shown in text-figure 1*A*. Closely similar data were obtained in both instances, indicating that the effects of these monovalent ions, though definite, were relatively small. The maximum rate of the reaction occurred close to a concentration of 0.05 *M* for both Na^+ and K^+, and the effect of Na^+ was slightly greater than that of K^+. The results of analogous experiments with the same plasma, describing the effects of the divalent ions Ca^{++} and Mg^{++}, are shown in text-figure 1*B*. These ions, acting individually, activated the reaction to levels not greatly different from those seen with Na^+ and K^+. The optimum concentration of the ions for activating the reaction was at about 0.04 *M*.



TEXT-FIGURE 1.—*A*. Influence of various concentrations of Na^+ and K^+ on the dephosphorylating activity of 0.5 ml. of plasma.

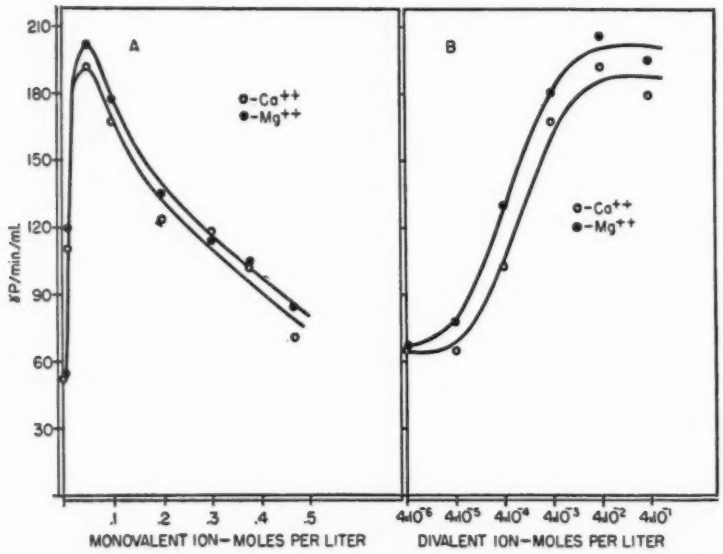
B. Influence of various concentrations of Ca^{++} and Mg^{++} on the dephosphorylating activity of 0.5 ml. of plasma.

Experiments were now made to determine the influence of mixtures of Na^+ and K^+ in various respective concentrations. It was found that the influence of the ions together was not greatly different from that of the ions acting separately, though a small quantitative interrelationship was apparent.[4] Here again the region of greatest effect was about 0.05 *M* concentration of each ion. The results obtained with mixtures of Ca^{++} and Mg^{++} were essentially similar to those observed with these ions acting separately; the divalent ions exerted their effects separately, neither one inhibiting nor potentiating the activity of the other.

In marked contrast, the activating effects of Ca^{++} and Mg^{++} were greatly enhanced by the presence of the monovalent ions. The influence of varying concentrations of KCl on the activating effects of optimum concentration, 0.04 *M*, of Ca^{++} or Mg^{++} is shown on text-figure 2*A*. The large effect of the monovalent ion on each of the divalent ions is apparent by a comparison of the curves of text-figure 2*A* with those of 1*B*. Similar results were obtained with Na^+. A study of a mixture of K^+ and Na^+, each in 0.05 *M* concentration, on Ca^{++} or Mg^{++} in varying concentration is shown in text-figure 2*B*. Here it is evident that the optimum concentration of both Ca^{++} and Mg^{++} for activation of the enzyme was close to 0.04 *M*.

[4] In the reaction mixture, $[Na^+] + [K^+] = [I]$ where [I] = total ion concentration.

If each ion acted separately the rate of the enzyme reaction, R_1, would be expected to lie between the rates with each ion studied separately. This relation was not observed over the range of concentrations investigated, nor was the rate an average (R_2) of the rates with Na^+ and K^+ used separately, each at concentration [I]. Instead, the rate, R_3, observed with mixtures of Na^+ and K^+, was the average of R_1 and R_2.

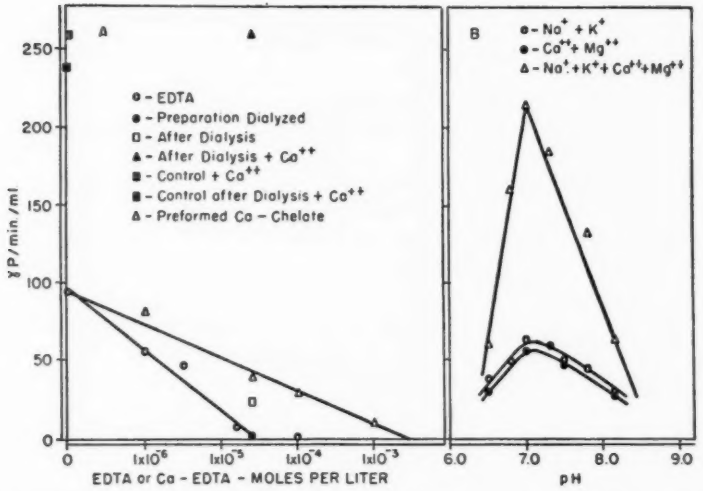


TEXT-FIGURE 2.—*A*. Influence of K^+ in various concentrations on the activating effects of constant concentrations, 0.04 *M*, of Ca^{++} and Mg^{++} on the dephosphorylating activity of 0.5 ml. of plasma.

B. Influence of the concentration of Ca^{++} or Mg^{++} in activating the dephosphorylating enzyme activity of 0.5 ml. plasma in the presence of a mixture of Na^+ and K^+, each 0.05 *M*.

Influence of versene.—The foregoing experiments have indicated the importance of ion effect on the activity of the dephosphorylating enzyme. It was of interest to study the phenomenon further with the use of versene which has the capacity to chelate quantitatively divalent ions. It was expected that such investigations might contribute to knowledge of the absolute effects of the divalent ions on the enzyme in plasma, inasmuch as all reaction mixtures will contain the amounts of Ca^{++} and Mg^{++} originally present in the plasma. Studies were made with 0.5-ml. volumes of plasma, a reaction medium of 0.05 *M* KCl at *p*H 7.0, and with adenosine triphosphate solution prepared in distilled water as before. The results obtained with this system on the inclusion of varying quantities of versene, dissolved in distilled water and adjusted to *p*H 7.0, are shown in text-figure 3*A*. There is seen a linear relation between the concentration of versene and the rate of the reaction under these conditions, and the activity observed with plasma alone, dependent on the calcium and magnesium naturally present in the plasma, was depressed almost to extinction.

Further information about the action of Ca^{++} and Mg^{++} was obtained by dialysis of a plasma preparation following the addition of versene. In this case an amount of versene was introduced sufficient to lower the enzyme activity of 0.5 ml. of plasma essentially to zero [the preparation



TEXT-FIGURE 3.—*A*. Influence of chelation of Ca^{++} by versene (EDTA) (open circles) on the dephosphorylating activity of 0.5 *M* plasma. The inhibiting influences of the preformed Ca-chelate (Ca-EDTA) is shown by the open triangles.

B. Influence of *p*H on activation of the dephosphorylating enzyme in plasma by monovalent and divalent ions.

indicated (closed circle) in text-figure 3*A*]. Immediately afterward, 1.0 ml. of the versene-plasma mixture was dialyzed for 18 hours against 2 liters of cold 0.05 *M* KCl solution. As a control the same volume of untreated plasma was dialyzed in the same way. In order to use the results with the control principally as an index of the effect of dialysis on enzyme action, the preliminary analyses on the control plasma were made in the presence of excess calcium, namely, in the balanced saline solution described under methods. The analysis with the control after dialysis was made under the same conditions. The findings, text-figure 3*A*, showed that with dilution by dialysis of the versene-Ca^{++} and versene-Mg^{++} complexes, the rate of enzyme reaction (open square) was very low but was still significantly higher than that observed before dialysis indicating a slight inhibiting effect of the chelates. In the control, dephosphorylation reached a level (closed square) about 10 percent lower than that (cross in square) of the control before dialysis, showing the lack of effect of dialysis either on enzyme activity or in the removal of hypothetical inhibitors. With the addition of Ca^{++} and Mg^{++} each in 0.04 *M* concentration to the dialyzed versene-treated preparation (closed triangle), the rate of enzyme reaction returned to the level exhibited by the control plasma before dialysis.

The inhibitory effect of the chelates of Ca^{++} and Mg^{++} was investigated further by the addition of preformed chelate to plasma assayed in 0.05 *M* KCl as the reaction medium. Under these conditions, the reaction rate declined with increasing amounts of the chelate, the curve of text-figure

3*A* indicated by open triangles, but the reaction failed to diminish to zero when the amount of chelate added was equivalent to the amount of free versene added in the previous experiment (open circle).

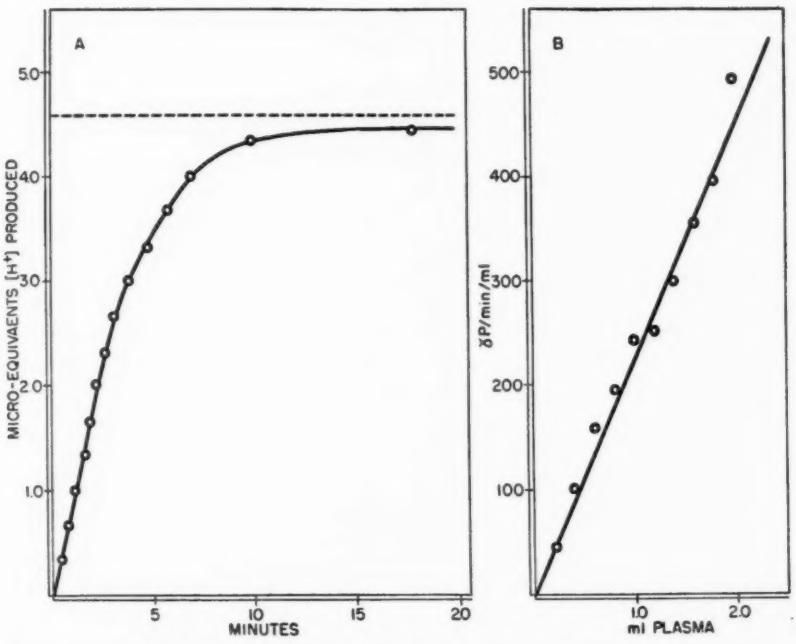
Influence of hydrogen-ion concentration.—Experiments were made to determine the effects of *p*H on the rate of the enzyme reaction in the various saline media as indicated in text-figure 3*B*. The range of null points employed was from *p*H 6.5 to *p*H 8.16, yielding the data of text-figure 3*B*. When the reaction was allowed to occur in saline solutions containing a mixture of 0.05 *M* K^+ and 0.05 *M* Na^+ the reaction rate was at a maximum at about 7.1 to 7.2, but the influence of *p*H was relatively small. Results similar in kind and extent were seen when only the divalent ions Ca^{++} and Mg^{++}, each in 0.04 *M* concentration, constituted the saline reaction mixture. In contrast, the influence of *p*H was great when both monovalent and divalent ions were present in the same concentrations as those employed separately. The *p*H optimum for the reaction derived from a series of such experiments carried out in the balanced saline solution was 7.16.

Nature and specificity of the dephosphorylation activity.—In the enzymatic dephosphorylation of adenosine triphosphate several possible reactions [5] might be suspected: 1) ATP→ADP + P; 2) ATP→AMP + P–P; 3) ADP→AMP + P; 4) 2ADP⇌ATP + AMP; 5) AMP→IMP + NH_3; and, 6) deamination of ATP or ADP and the production of adenosine from adenylic acid.

The problem was approached first by the estimation of the amount of phosphorus liberated in the reaction with a measured quantity of adenosine triphosphate. For this the adenosine triphosphate in distilled water, 7.39 micromoles of intact adenosine triphosphate in 0.02 ml., determined as described above, was added to 0.5 ml. of plasma in a saline reaction solution of 0.15 *M* KCl at *p*H 7.0. The reaction was followed to completion as shown in the curve of text-figure 4*A*, yielding 4.42 microequivalents of acid. An aliquot of the final reaction mixture, 8.5 ml., was deproteinized with trichloroacetic acid, and a portion of the filtrate resulting from removal of the protein was assayed for inorganic phosphorus (*17*). The amount of phosphorus produced, 7.23 micromoles, corresponded closely with that predicted on the assumption that the terminal phosphate of the adenosine triphosphate was completely hydrolyzed. Calculations from these data indicated an equivalence ratio $\frac{(H^+)}{P}$ of 0.61. This is closely comparable with the analogous value, 0.62, obtained in the study of myosin (*20*) under identical conditions.

An equal aliquot of the same reaction mixture was treated with perchloric acid, and the protein precipitate was removed by filtration. By addition of KOH, the *p*H of the filtrate was brought to 8.5, the preparation was cooled to 4° C., and the precipitated potassium perchlorate was removed by centrifugation. The supernatant fluid was diluted to

[5] ATP = adenosine triphosphate; ADP = adenosine diphosphate; AMP = adenylic acid; P = inorganic phosphate; P–P = inorganic pyrophosphate; and IMP = inosinic acid.



TEXT-FIGURE 4.—*A*. Course of the dephosphorylating reaction under optimum conditions. The dash line indicates the theoretical yield of acid from the amount of adenosine triphosphate employed.

B. Relation of dephosphorylation to the volume of plasma employed.

10 ml., and then 1.0-ml. volumes of the fluid, diluted again to 10 ml., were passed over a column of Dowex-1 (in chloride cycle), and the respective nucleotides were successively eluted by 10-ml. volumes of the respective solvents employed in the method of Cohn and Carter (*18*). Quantitative estimation of the individual nucleotides was made with the Beckman spectrophotometer. In addition a complete spectrum was obtained for each fraction. There was found a trace only of the triphosphate; more than 95 percent of the nucleotide content of the preparation consisted of adenosine diphosphate, an amount that was the molar equivalent of the phosphorus already determined in the first aliquot. The absorption spectrum of the diphosphate fraction was at a sharp maximum at 2,600 Å, indicating that no significant deamination had occurred. The results of these experiments indicate that the only reaction occurring consisted in quantitative hydrolysis of the terminal phosphate of the adenosine triphosphate and the absence of the complications of hydrolysis of adenosine diphosphate or its dismutation ($2ADP \rightleftharpoons ATP + AMP$) by the intervention of myokinase. Inasmuch as adenylic acid was not produced, the system provided no information about the occurrence of adenylic acid deaminase activity in the plasma. This possibility was tested by setting up a system similar to that above except for the substitution of

adenylic acid for adenosine triphosphate and the use of 0.001 *M* Ca^{++} in the reaction mixture in place of the 0.04 *M* concentration of Ca^{++} and Mg^{++}. After incubation of the mixture for 3 hours at 30° C., an aliquot was deproteinized with perchloric acid, and the potassium perchlorate was removed as before. Spectrophotometric examination of the supernatant fluid revealed a maximum absorption at 2,600 Å, indicating no deamination in the reaction mixture.

Relation of enzyme activity to plasma volume.—The association of enzyme activity with different volumes of plasma is shown in text-figure 4*B*. This experiment was made under the optimum conditions described in the section on methods with adjustment of the volume of the saline reaction mixture in accordance with the volumes of plasma employed as indicated in text-figure 4*B*. The relation observed was linear over a tenfold range of plasma dilution, which was the result expected of an enzyme reaction.

Discussion

It is seen from the results of the present work that the dephosphorylation of adenosine triphosphate by the plasma of chickens with erythromyeloblastic leukosis is a typical enzymatic activity. Experiments reported earlier (*2*, *5–7*) have provided a substantial foundation for the concept that this enzyme activity is due either to an integral constituent of the virus or to a material so intimately associated with it that virus and enzyme occur simultaneously and proportionately and cannot be separated by washing, by sedimentation, or by electrophoresis. In the face of these findings and in the absence of any evidence by electron micrography of a significant secondary population of particles associated with the virus, it has appeared reasonable to assume with considerable assurance that the enzyme is a component of the virus.

With this assumption, the enzyme is characterizable with some clarity. It occurs in association with a particulate material (*3*, *4*) found in variable amounts in the plasmas of birds diseased with erythromyeloblastic leukosis. The physical properties of the particles have been established by electron micrography (*3*, *4*), sedimentation behavior (*6–8*) and electrophoretic mobility (*6*, *7*). These particles likewise constitute the virus of the disease, displaying the specific capacity to induce the condition in the susceptible host. The action of the enzyme, with respect to adenosine triphosphate, is specific and limited, *in vitro*, to the hydrolysis of the terminal phosphate of this substrate. Under optimum conditions hydrolysis closely approaches the theoretical endpoint, yielding free phosphate and adenosine diphosphate in equivalent amounts. Adenosine diphosphate is unaffected either by hydrolysis or dismutation, and there was no effect on adenylic acid introduced into the system.

The activity of the enzyme is strongly influenced by ion kind and concentration. Activity is enhanced to small degree by K^+ and Na^+ acting alone or together, and the same was true with Ca^{++} and Mg^{++}. In contrast both Ca^{++} and Mg^{++}, separately or together in the presence of either or both K^+ and Na^+ strongly activated the reaction. In this

respect the optimum molar concentrations of Ca^{++} and Mg^{++} were essentially the same, and both acted in similar degree. Chelation by versene eliminates the effects of either Ca^{++} and Mg^{++}. The influence of *p*H is definite and large, there being a single sharp optimum at *p*H 7.16. This effect is small unless both divalent and monovalent ions are present.

It would be very helpful, if it were advisable, to employ the present data as a biochemical foundation for further interpretations with respect to the relation of the enzyme to the virus. Indeed, reasoning by analogy from findings with tissues of diverse origin (*21–25*), it would be possible greatly to restrict the problem of the nature of nonviral materials which might be present in the plasma to contribute to the adenosinetriphosphatase activity. It is evident that the virus adenosinetriphosphatase in its specificity, ion activation, *p*H optimum of activity, stability (as will be reported later), and other properties is unique among the known analogous enzymes of tissue origin. With the information already at hand, there can be eliminated conclusively participation in the enzyme activity of any known derivatives of chicken red blood cell constituents (*22*). A similar decision could be made in relation to the presence of mitochondria and mitochondrial fragments from the primitive white cells if these structures could be assumed to possess the quantitative and qualitative properties of the components of other cells such as those from the liver (*24*), for example. Nevertheless, such assumptions are not justified in view of known radical differences (*22*) in the properties of cell structures of diverse origin and especially so since primitive cells are available in plenty for direct study. The various differences and the similarities to be seen among the adenosinetriphosphatases, as well as in other types of enzyme activity of cellular material, do suggest, however, a means of approach to the question of the specificity of the adenosinetriphosphatase activity seen here with the virus particles. These possibilities have indicated the direction of investigations next to be considered in this field.

Summary

Studies have been made on the properties of the adenosinetriphosphatase activity associated with the virus of erythromyeloblastic leukosis in the blood plasma from diseased chicks. The behavior of the material was typically that of an enzyme. Dephosphorylation was proportional to the volume of virus-containing plasma studied, and the activity was specific and limited to hydrolysis of the terminal phosphate of the substrate. Under suitable conditions hydrolysis approached closely the theoretical endpoint yielding equivalent amounts of free phosphate and adenosine diphosphate. The latter product was unaffected by hydrolysis or dismutation, and no alteration was observed in adenylic acid added to the system. Dephosphorylation was activated equally by Ca^{++} or Mg^{++}, separately or together, in the presence of either or both K^{+} and Na^{+}. Chelation with versene eliminated the effects of the divalent ions. There was a single sharp peak at the *p*H optimum of activity at 7.16.

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Serologic Activities of Serum Fractions in Animal Neoplasia[1]

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We have reported previously (*1*, *2*) that the sera from most rabbits carrying the Brown-Pearce carcinoma gave positive reactions when tested by a quantitative 50-percent hemolysis, complement-fixation method, using saline extracts of the neoplasm as antigens. In order to determine the types of serum proteins with which this complement-fixing activity was associated, pools of sera from normal rabbits and from animals carrying the neoplasm (designated in this report as tumor serum) were fractionated by the use of ethanol, and the resulting fractions were tested by the same complement-fixation method that had been developed for testing whole serum.

Nichol and Deutsch (*3*) have described a rather simple ethanol fractionation method for preparing a relatively pure precipitin-rich *gamma*-2 globulin fraction (precipitate C) from immune rabbit serum. We followed their method with a slight modification (text-fig. 1). The crude globulins were separated initially as precipitate A and this precipitate was subfractionated to yield the fairly pure *gamma*-2 globulins of precipitate C plus the remaining globulins of precipitate B. In addition, Method 6 devised by Cohn and his associates for the fractionation of human-plasma proteins (*4*) was used to subfractionate the crude albumin of supernatant A in order to prepare the purified albumin of precipitate E. The fractions were analyzed electrophoretically, and their average compositions, approximately the same for normal or tumor serum, are also shown in text-figure 1. All fractions, crude or purified, were tested for their complement-fixing activity, but those studied most intensively were the purified albumin, precipitate E, the *gamma*-2 globulins of precipitate C and the mixture of *alpha*, *beta*, and *gamma*-1 globulins of precipitate B.

We had found previously (*5*) that the temperature at which whole serum was inactivated influenced considerably the amounts of complement required for 50-percent hemolysis in its presence (its anticomplementary properties) and the degree of complement fixation obtained in the presence of antigen. Therefore, although the serum fractions did not require inactivation by heat in order to destroy complement, it seemed advisable to

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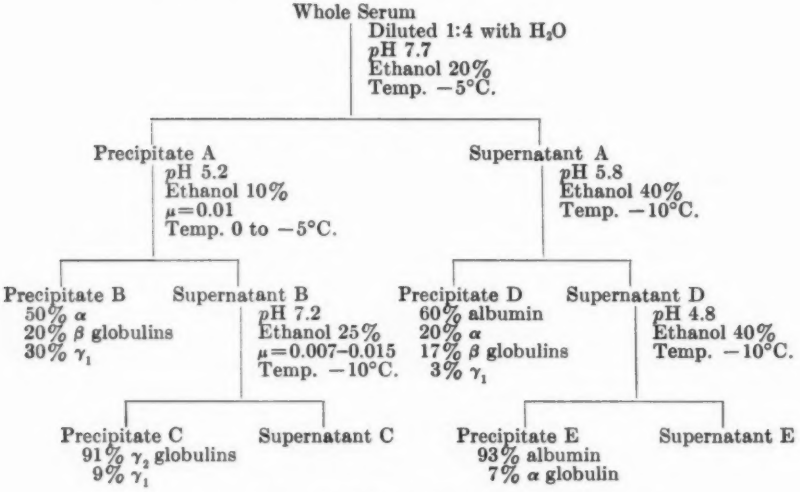
investigate the effects of the same inactivation temperatures that had been used for whole serum upon the anticomplementary properties of the various serum fractions. The fractions, unheated and heated for 30 minutes at 56, 61 and 65° C., were tested in the absence of antigen.

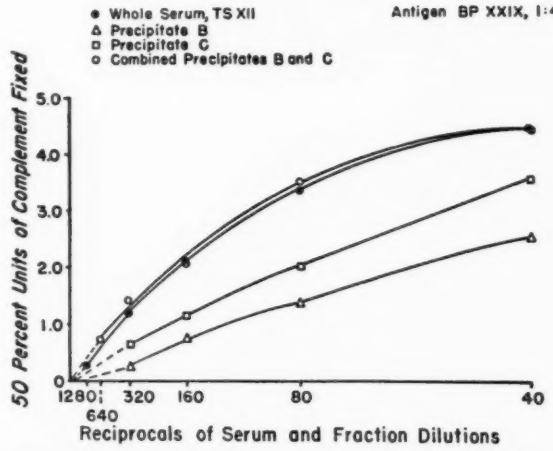


TEXT-FIGURE 1.—Alcohol fractionation of rabbit serum.

The albumin fractions were not anticomplementary when tested either unheated or heated at 56, 61 or 65° C. In each case precipitate B was extremely anticomplementary when tested unheated but there was little evidence of this property after it was heated at any of the three temperatures. In each case precipitate C showed little or no anticomplementary property when tested unheated or after heating at 56° C., but it was definitely anticomplementary and gave atypical hemolysis curves after heating at 61 and 65° C. Consequently complement-fixation tests were performed on these fractions under conditions giving the least interfering anticomplementary activity and requiring the smallest amount of heating. The albumin fractions (supernatant A and precipitate E) and the *gamma*-2 globulins (precipitate C) were tested unheated, while precipitate B, the other protein fractions, and the corresponding sera from which the fractions were prepared were tested after heating at 56° C.

In our complement-fixation procedure the number of 50-percent units of complement fixed by a tumor serum is determined by using a pool of normal rabbit serum as the baseline. Therefore, the amount of complement fixed by each fraction or combination of fractions was determined by use of the corresponding normal rabbit-serum fraction or combination of fractions as the baseline. The curves in text-figure 2 show the comparative complement-fixing activities of one of the pools of strongly positive tumor sera (TS XII) and some of its fractions when saline solutions of the fractions were diluted so as to be comparable with serial dilutions

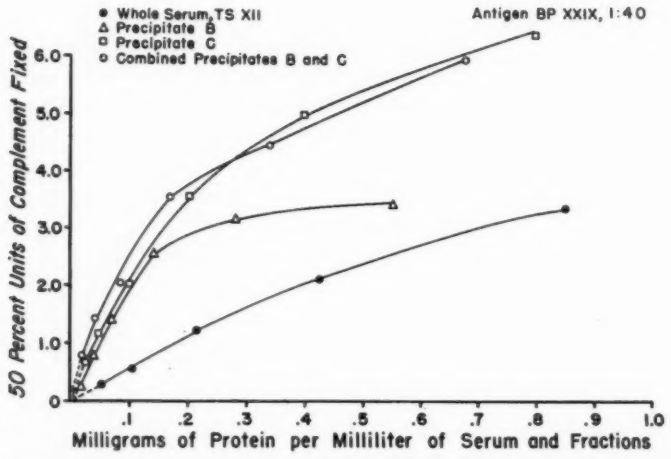
of the original tumor serum. The antigen used was a saline extract of the Brown-Pearce carcinoma. The 50-percent units of complement fixed have been plotted against the dilutions of TS XII and its fractions. The resulting activity curves tended to be linear at the higher dilutions of the whole tumor serum or its fractions. Therefore, the relative activities of the different preparations were estimated at these higher dilutions by comparing the dilution of whole tumor serum that fixed a given number of complement units with the dilution of the given fraction or combination of fractions that fixed the same number of complement units.



TEXT-FIGURE 2.—Complement-fixing activity of TS XII and its fractions on a dilution basis. Average values of 4 determinations.

On this dilution basis neither the *gamma*-2 globulin of precipitate C nor the mixture of *alpha*, *beta* and *gamma*-1 globulins of precipitate B was as active as the original serum; the *gamma*-2 globulin had 50 percent and the mixture of remaining globulins, 30 percent of the activity of the whole tumor serum. However, the combination of these two fractions was approximately as active as the original whole serum. In addition, precipitate D had approximately 12 percent of the activity of the whole tumor serum. Supernatants C and E were inactive. Results similar to the above were also obtained in tests of TS #ACI and its fractions.

Text-figure 3 shows the same data given in text-figure 2, but they are expressed in terms of the protein concentration of TS XII and its fractions at each dilution tested. Since the 2 globulin fractions represented only a small proportion of the total serum protein, they fixed more complement per unit (0.1 mg.) of protein than did the whole serum. On this basis, therefore, the *gamma*-2 globulin fraction and the mixed *alpha*, *beta* and *gamma*-1 globulin fraction were each approximately 5 times as active as the whole tumor serum, and the combined globulins (precipitates B plus C) represented a sixfold concentration of the activity of TS XII.



TEXT-FIGURE 3.—Complement-fixing activity of TS XII and its fractions on a protein basis. Average values of 4 determinations.

TABLE 1.—*Relative activity of serum fractions*

Serum or fractions	Temperature of inactivation	No. of preparations or serum samples tested	Average activity as percent of activity of whole TS at same dilution (1:10)	Average protein concentration in mg./ml. of 1:10 dilution	Average activity per unit of protein as percent of activity of whole TS at same dilution (1:10)
Whole tumor serum. (TS)	56°C.	4	100	6. 23	100
Supernatant A..... Crude albumin	unheated	4	9	3. 6	16
Precipitate E..... Averaged 93% albumin 7% α globulin	unheated	2	2	1. 6	8
Precipitate B..... Averaged 50% α globulin 20% β globulin 30% γ_1 globulin	56°C.	4	46	0. 60	480
Precipitate C..... Averaged 91% γ_2 globulin 9% γ_1 globulin	unheated	4	50	0. 61	510

Table 1 summarizes the results obtained with 4 series of fractions, each series being obtained from a different pool of reactive tumor serum. The average activities of the *gamma*-2 fraction (precipitate C) and of the mixed *alpha, beta* and *gamma*-1 fraction (precipitate B) each represented about 50 percent of the average activity of the whole TS pools at the same dilution, but in terms of protein concentration they each represented about 500

percent of the average whole TS activity. The crude and even the purified albumin fractions represented a considerable proportion of the serum proteins, but they were nearly inactive.

Conclusions

We have concluded that the complement-fixing activity of sera from rabbits bearing the Brown-Pearce carcinoma is associated with the serum globulins. Approximately one half of the activity was found in precipitate C which contained about 91 percent *gamma*-2 and 9 percent *gamma*-1 globulins. Most of the remaining activity was found in precipitate B, which contained 50 percent *alpha*, 20 percent *beta* and 30 percent *gamma*-1 globulins.

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Mitotic Poisoning by 4-Aminostilbenes[1,2]

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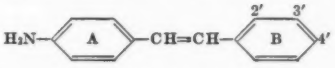
Using the Walker rat carcinoma 256, Haddow and co-workers (*1*) determined the relationship between the growth-inhibitory effect and the chemical constitution of various derivatives of 4-aminostilbene. In order to throw light on the cellular basis of growth-inhibition, the author has undertaken an analysis of the mitotic abnormalities which appear after the administration of the drugs. The investigation disclosed many phenomena of mitotic poisoning and brought to light facts which are thought to have interesting bearing on the biological mechanism of drug action on malignant cells. The present paper describes the moiety of mitotic disturbances and discusses their significance and relationship to growth-inhibition.

Materials and Technique

The basic structure of the aminostilbene molecule consists of two benzene rings linked together by an ethylenic double bond.



It was used as the parent-molecule from which a great variety of aminostilbenes were derived. The most important is 4-dimethylaminostilbene, the hydrogens of NH_2 in ring A having been replaced by two methyl groups.

The cytologic effects of 4-amino- and 4-dimethylaminostilbene and several derivatives of the latter were analyzed. In these, chloro, methyl, fluoro and bromo substituents were placed at different positions in the benzene ring—B. The compounds were prepared by Haddow *et al.* (*1*).

The substances were injected intraperitoneally into rats as suspensions

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[3] The author is greatly indebted to Professor A. Haddow for the help he has given during the investigations, and thanks Mr. J. L. Everett, who has prepared most of the suspensions used in the experiments.

in sterile arachis oil. Control experiments showed no effects when 1 to 2 cc. of arachis oil was injected alone. The relatively high toxicity threshold of the aminostilbenes made it possible to study the cytologic effects of doses within a wide range. The dose varied in different experiments from 25 mg. to 550 mg. per kg. of body weight. The systemic toxic effects—among which the loss of weight is the most important—were first noticed on the 5th day after treatment.

Throughout the experiments albino rats of Wistar origin were used; their age varied from 5 to 8 weeks and their weight from 160 to 210 grams. The Walker carcinoma 256 was grafted into the right flank and the various compounds were injected 5 days later. Because of the variable growth-rate shown by the grafts (*2*), the animals to be treated had been selected for tumors of the same size. The tumor-bearing rats were killed at 8, 12, 18, 24, 48, 72 and 96 hours after administration of the drugs. Walker carcinoma was used throughout the experiments, except where otherwise stated.

For cytologic analysis small pieces of the tumor were fixed in acetic alcohol and squash preparations were made, stained with acetic-lacmoid or Feulgen's basic fuchsin (*3*). By using this method the characteristic features of mitosis are distinct and can be analyzed (figs. 1 to 4). The squash method is very convenient to identify the disturbances which appear in dividing cells after the administration of the various aminostilbenes. The histologic changes have been studied on sections stained with hematoxylin and eosin.

The Morphology of Mitotic Abnormalities

After the administration of aminostilbenes a wide range of abnormalities was observed in the dividing cells of the Walker carcinoma. The mitotic disturbances produced by different members of this group of substances differ in frequency and time of onset. On the other hand, they are qualitatively very similar and can be grouped into three classes, each having a definite time sequence in its development. The characteristic features of these classes are described below.

Primary or "Physiological" Effects

The first abnormalities may appear as soon as 8 to 10 hours after injection of certain aminostilbenes (*e.g.*, 4′-fluoro-4-dimethylaminostilbene) but, on the average, their time of onset is 16 to 18 hours—when the dose is not higher than 80 mg. per kg.

The most common effect is chromosome "stickiness" (fig. 5). This term is used to describe the cause of the configuration which is brought about without indicating the chemical basis of this phenomenon. Chromosomes may stick together along their whole length, though commonly stickiness is restricted to the distal ends. When the proximal parts of chromosomes (where the centromere or spindle-fiber attachment is located) move toward the poles, the result is a configuration similar to that of meiotic bivalents (fig. 6) (*4*).

In the early tumor samples (6 or 7 days after implanting) cells showing "clumping" of chromosomes at metaphase were frequent, and this was usually associated with a delay in spindle formation. In later tumor samples (8 to 10 days after implanting), relatively few degenerating cells were found with clumped chromosomes; therefore, it may be assumed that metaphase clumping is a temporary phenomenon.

Cells with dumbbell-shaped nuclei and cells with nuclear bodies are two other abnormal cell types, which have frequently been seen in tumor samples taken between 10 to 24 hours after treatment. The development of dumbbell-shaped aneuploid nuclei (fig. 7) is due to prolonged postmetaphase stickiness of a large number of chromosomes within the same cell. The "chromosome bridges," which appeared during anaphase and telophase, prevented the separation of the two daughter nuclei (fig. 8). Early tumor samples contained many cells showing the intermediate stages of this process.

The other abnormal cell-type lacked an organized nucleus and contained several "nuclear bodies" of varying sizes, all giving a Feulgen-positive reaction (fig. 9). For that reason they were assumed to be chromosome material; the number of the chromosomes determines the size of each group. This abnormality is described in the older literature as "karyorrhexis." The nuclear bodies were usually scattered at random, but in some cells their position suggested the presence of an incomplete spindle. It seems that a general postmetaphase sticky state of the chromosomes and the absence of the mitotic spindle were responsible for the appearance of nuclear bodies, and the process may be described as anaphase pyknosis.

With few exceptions, many of these effects (particularly stickiness of chromosomes and dumbbell-shaped compound nuclei) have been found in nontreated Walker carcinoma as well as in many other malignant growths (*5*), and are especially frequent in necrotic tumor regions. The cause underlying this type of mitotic disturbance has been discussed by the author in a previous paper (*4*).

The abnormalities just described are very similar to those found in cells after irradiation (*6*) and nitrogen-mustard treatment (*7*). These studies led to the conclusion that the abnormal chromosome behavior is due to disturbances in the cytoplasmic environment of the cell. Marquardt (*8*) described them as the primary or "physiological" effects of irradiation. They appeared a few hours after treatment with X rays or chemical agents indicating that the cells showing the abnormalities were in mitosis when irradiated or when the drug was absorbed.

It is known that during mitosis special cytoplasmic environment (*e.g.*, viscosity) is required for the organization of the spindle (*9*). The cytoplasmic viscosity is altered by X rays or drugs (*10*), and the change may be responsible for the absence of the spindle and stickiness of chromosomes in the affected cells. Because the primary anomalies can be attributed to disturbances in the cytoplasm they may be referred to as "pyhsiological" effects.

It has been found that the incidence of cells with naturally occurring mitotic abnormalities in the Walker carcinoma does not rise above 5 per-

cent. In view of the fact that in tumors treated with aminostilbenes much higher frequencies were found, it may be inferred that the mitotic disturbances were the result of the treatment.

Neucleotoxic or Radiomimetic Effects

In the tumor samples taken at 24 or 48 hours after the administration of the aminostilbenes, dividing cells appeared with fragmented chromosomes. If the fragments lie off the equatorial plate they can be seen as early as metaphase (fig. 10), though they are most easily recognized in the postmetaphase stages. Because the centromere is necessary for the active movement of the chromosomes, the broken chromosome segments without a centromere (*acentric fragments*) are left in the cytoplasm between the two polar groups of daughter chromosomes (figs. 11 and 12).

Extensive studies of chromosome injuries in X-rayed cells led to the conclusion that the initial damage, which causes the fragmentation of the chromosomes, takes place in the nucleus during interphase (*11*). The double structure of acentric fragments seen at metaphase (fig. 13) is evidence that the breaks occurred before the duplication or "splitting" of the parental chromosome thread has taken place. The broken ends of the acentric sister chromatids can rejoin; the ring- or V-shaped fragments are the result of such a fusion (figs. 11, 12, 14).

Cells with single chromosome bridges were also found. The bridges seen in anaphase and telophase are the result of fusion between those ends of the broken chromatid segments that contain the centromere (fig. 15). The presence of double bridges with acentric fragments indicates that translocation, *i.e.*, fusion between broken chromosomes, also occurs. The higher frequency of cells with acentrics to that of cells with bridges shows that sister-reunion of acentrics is more common than that of the centric portion of broken chomosomes.

In several nondividing cells supernumerary nuclei of varying sizes and numbers were observed (fig. 16). They are called *micronuclei* and represent acentric chromosome fragments which have been left in the cytoplasm during the previous mitosis. While the very small micronuclei (0.5–1.5 mμ in diameter) have no visible structure—the larger ones show some internal organization. Cells with micronuclei usually degenerate, but occasionally they may divide, show bridges and acentrics (fig. 17), and break down early during the process (fig. 18). It may be of some interest to note that tumor cells that enter mitosis in the presence of micronuclei are always lying adjacent to normal cells undergoing mitosis.

Cells were also found which contain a large dumbbell-shaped nucleus and a variable number of micronuclei (fig. 19). Their presence in the cell indicates that the abnormal nucleus is the consequence of chromosome breakage which is followed by fusion of the broken chromosomes. The dumbbell-shaped nucleus thus formed differs from that which is due to chromosome stickiness (*cf.* figs. 7 and 19).

It has already been mentioned that chromosome fragmentation, postmetaphase bridges, and micronuclei represent injuries to the chromosomes

initiated during the interphase. At that time the chromosomes are within the nucleus, hence the injuries described above can be classified as *nucleotoxic* effects to indicate the place of their origin. Because these abnormalities are similar in appearance to chromosome injuries seen after irradiation, they may also be referred to as *radiomimetic* effects. It is necessary to emphasize that the adjective indicates only a similarity between the "end-products"; thus the primary mode of action of methyl bis(2-chloroethyl)amine (HN_2) by which injuries are inflicted on the chromosomes, has been found to be different from that of X rays (7).

The present investigation has shown that the frequency of cells with nucleotoxic or radiomimetic injuries is related to concentration, dose, and duration of treatment. It was furthermore established that the incidence of the abnormally dividing cells is affected by chemical substitution in the aminostilbene molecule. Thus it became evident that the radiomimetic effects could be used as criteria to compare the biological activity of different aminostilbene derivatives (figs. 11 and 12). Investigation was undertaken in this respect and the data obtained are presented in the second part of this paper.

Complex or Secondary Cytotoxic Effects

The length of the period during which the carcinoma cells show only radiomimetic effects is variable; it may be restricted to a few hours or it may spread over a long period. When the dose is not higher than 50 mg. per kg., the number of abnormal cells showing radiomimetic injuries is at a maximum at 24 hours and the effects disappear at 72 hours after treatment. By increasing the dose to 150 to 200 mg. per kg., it was noticed that the frequency of abnormal cells increased but the injury within individual cells became complex—very often obscuring the character of true radiomimetic effects.

The type of the cytologic damage suggests that both chromosomes and cytoplasm are affected. Thus cells with spindle abnormalities were particularly frequent. The "hollow" spindle, which has a very large diameter and spreads through the whole cell, is such an abnormality (fig. 20). Cells with abnormally thin or thick chromosomes were also found. The thin, long, lightly staining chromosomes seen in the equatorial plates of many dividing cells are the result of faulty spiralization. On the other hand, the short, thick, bulky chromosomes indicate supercontraction of the chromosome filament. The chromosomes are usually scattered throughout the cytoplasm because the spindle mechanism is absent (fig. 21). Figure 22 illustrates one dividing cell with a hollow spindle (side view), and a second one with superfragmented chromosomes. Cells were observed that were filled with fragmented chromosomes (fig. 23). If the broken chromosomes fuse together, nuclear bodies are formed (fig. 24). Cells with such bodies were very numerous in tumor samples, taken at, or later than, 72 hours after treatment.

In the late tumor samples the excessive chromosome fragmentation and severe spindle abnormalities made the analysis of cell injuries very

difficult (fig. 25). The low incidence of dividing tumor cells on the one hand, and the fact that many nondividing cells also exhibit degenerative changes on the other, further complicated the study of induced effects. For these reasons, the cytologic analysis had to be restricted to tumors that were treated for periods up to 72 hours. During that period the secondary cytotoxic effects were usually absent in tumors treated with various aminostilbenes.

Histologic Alteration in the Walker Carcinoma

The secondary cytotoxic effects which appear after the administration of the aminostilbenes are accompanied or closely followed by changes in the histologic structure of the Walker tumor (figs. 26 to 29). Similar changes were observed in the same carcinoma after treatment with HN_2 (*7*). By analyzing the initial stages of this process, it was found that the histologic alteration can be attributed to the increased proliferation and differentiation of the "histiocytes." These particular cells were primitive fibroblasts of the host and appeared during the local reaction after the grafting of tumor into subepidermal tissues (*7*, *12*). The histiocytes by their smaller size can be easily identified during mitosis (fig. 28). It was observed that their number decreases with the age of the tumor: in a 10-day-old Walker tumor the histiocytes represent only about 5 percent of the cell population; in 14-day-old tumors these cells are almost completely absent.

While in 6- to 8-day-old control tumors dividing histiocytes are rather rare, it was found that in tumors of the same age, which had been treated with aminostilbenes 72 or 96 hours previously, a high number of cells in mitosis belong to the histiocyte class (table 1). It was also observed that many dividing histiocytes had nucleotoxic and cytotoxic effects, but the extent of these injuries was always less than was found in the tumor cells. Because of the higher mitotic rate and the lower incidence of ab-

TABLE 1.—*The number of dividing tumor cells and histiocytes in the Walker carcinoma** (*50 dividing cells are classified*)

Substances (*intraperitoneal injections*)	Dose† (*mg./kg.*)	Number of dividing cells after treatment (*in hours*)			
		72		96	
		Tumor cells	Histiocytes	Tumor cells	Histiocytes
4-dimethylaminostilbene	120	28	22	13	37
Derivatives:					
2′-chloro	150	23	27	9	41
2′-methyl	150	17	43	16	34
Control‡	———	47	3	43	7

*Age of tumor at the time of injection: 5 days.
†Average weight of rats: 180 grams.
‡The tumor-bearing rats were injected intraperitoneally with 1 cc. of arachis oil only.

normal mitosis the histiocytes soon outnumbered the tumor cells (fig. 27). This process is followed by the development of an extensive collagenous matrix, in which are scattered the tumor cells. Most of these tumor cells show degenerative nuclear changes (figs. 29 and 30).

Mitotic Poisoning and Growth-Inhibition

By comparing the cytologic and histologic effects induced by the various aminostilbenes it was found that the growth of the Walker tumor could be arrested in cases only when not less than 70 percent of dividing tumor cells were injured within 72 hours of the administration of the drugs. It was observed, furthermore, that the dose which could produce that amount of injury varied for the different aminostilbene derivatives, and that the growth of the Walker tumor could be inhibited more easily if the transplanted tumor was not older than 5 days. Thus, in a 9-day-old tumor, in spite of the fact that 70 percent of the dividing tumor cells had been injured by administering particular aminostilbenes, the growth inhibition was of very short duration. The same dose of the same aminostilbenes, on the other hand, was found to be a very effective growth-inhibitor when the tumor was only 4 days old. A similar observation was made by Hendry *et al.* (*13*) when testing the growth-inhibitory property of methylolamides on the Walker tumor.

It has been found that in "young" tumors (4–6 days old) the mitotic rate is high and histiocytes are numerous, while in "older" ones (7–12 days old) dividing cells are less frequent and histiocytes are almost entirely replaced by tumor cells. It seems that in order to bring about a complete stoppage of growth the primary cytotoxic and nucleotoxic injuries in the tumor cells must be combined with an increased activity of histiocytes.

Experiments in which fractionated treatment was used support this assumption. When 2 or 3 doses of aminostilbenes have been given at 24-hour intervals, inhibition of growth was only temporary. The Walker tumor which resumed its growth, showed no histologic changes, indicating that the activity of histiocytes was insufficient to cause "fibrosis." Our investigation demonstrates that the histologic changes, as well as the cytologic injuries, are both necessary to stop the growth of Walker tumor.

The effects of various aminostilbenes have also been studied on two rat sarcomas (CB 1047 and CB 2041). The first was induced by repeated subcutaneous injections of *N,N*-di-2-chloroethyl-α-naphthylamine and the second by 1,4-dimethanesulphonyloxybutane; both were maintained by transplantation through many generations. Both tumors are fibrosarcomas and differ histologically from the Walker tumor. Table 2 shows the percentage of injured tumor cells 24 and 48 hours after the administration of the same dose of 2′-chloro-4-dimethylaminostilbene. It was observed that the frequency of injured cells is somewhat less in these tumors than was obtained in the Walker. The slight reduction, however, is not sufficient to account for the fact that in both sarcomas only partial inhibition of growth could be obtained, even in cases in which the highest

sublethal dose was administered. The poor response to aminostilbenes can chiefly be attributed to the absence of histiocytes.

TABLE 2.—*The frequency of injured cells in different tumors after 2′-chloro-4-dimethylaminostilbene (150 mg./kg.)*
(100 dividing cells are analyzed in each tumor)

Tumors	Percentage of injured cells at given hours after treatment	
	24	48
Walker carcinoma 256	17	42
CB 1047 sarcoma	10	31
CB 2041 sarcoma	13	27
20-methylcholanthrene—primary sarcoma	7*	10

*In another 20-methylcholanthrene-induced primary tumor, abnormally dividing cells were found without any treatment (8 percent).

Besides the transplantable sarcomas CB 1047 and CB 2041, the reaction of several primary fibrosarcomas to the aminostilbenes was also studied. These tumors were induced by subcutaneously implanted 3,4-benzpyrene pellets (0.5 mg.) and by subcutaneous injections of small doses of 20-methylcholanthrene in arachis oil. While both nucleotoxic and cytotoxic effects were observed in these sarcomas after the administration of 2′-chloro-4-dimethylaminostilbene (150 mg./kg.), their rate of growth was not altered. It may be emphasized that the histologic structure of these primary tumors was similar to that of CB 1047 and CB 2041.

These observations led us to conclude that mitotic poisoning is only the first stage in growth-inhibition, and must be accompanied by definite histologic changes to bring about a permanent, or at least a prolonged inhibition, in particular tumors. If there is no histologic response, as is the case in the various fibrosarcomas tested, growth-inhibition is slight and of short duration. In the Walker tumor, under certain conditions, both cytologic and histologic changes can be induced which result in inhibition of growth. For that reason this tumor is a suitable experimental object for testing the biological activity of particular chemical agents, when the criterion of activity is growth-inhibition. Thus, the "Walker-tumor test" has demonstrated the growth-inhibitory effects of methyl bis(2-chloroethyl) amine (*7*), ethylenimines,and diepoxides (*14*) and dimethanesulphonyloxybutane (*15*), while the effects of these drugs on other tumors were found to be nil or very slight.

Differences in Cell-Sensitivity

The duration of the interphase in the active cells of the Walker tumor has been estimated to be 32 hours (*16*). Data of table 5 show that the injury in dividing cells is greater 48 than 24 hours after treatment, which suggests that cells at the beginning of interphase are more sensitive to the action of aminostilbenes than those treated at the end of the inter-

mitotic period. It has already been reported that after treatment with HN_2, the most sensitive period in this tumor is the early phase of the intermitotic stage (*7*). Experimental evidence has been presented by Revell (*17*), which again shows that after treatment with diepoxypropyl-ether the most sensitive period in the meristem cells of *Vicia* is the early interphase.

The cause of the greater cell-sensitivity to drug action during the early intermitotic period may be manifold. It can be due to difference in the concentration of the drug within the cell at different stages of the mitotic cycle. This may be brought about by the particularly high permeability of the cell membrane during the beginning of interphase (*18*) or by the longer interval which elapses between treatment and taking the tumor sample, allowing a greater accumulation of the drug within the cell. When the chemical agents have very low solubility, the latter cause may be the most likely explanation. However, the difference in cell sensitivity may be due simply to the fact that metabolic activity, particularly that which is directly connected with chromosome synthesis, is most intensive during early interphase and damage in this stage has the most serious repercussions (*10*).

During the present investigation it was observed that in the same tumor the frequency of injured histiocytes and the amount of injury per cell in the histiocyte class was less than in the tumor cells. The lower sensitivity of histiocytes may be correlated with the longer mitotic cycle. The difference in sensitivity to the aminostilbenes shown by the two types of cells is the most important factor conditioning growth-inhibition in the Walker tumor.

It was also found that the malignant cells of different tumors differ in sensitivity when exposed to the same treatment (table 2). The greatest sensitivity was shown by the cells of the Walker carcinoma. Somewhat less sensitivity was shown by fibrosarcoma CB 1047 and CB 2041. It is of some interest to note that the malignant cells of these sarcomas are fibroblasts, which in many respects correspond to the histiocytes seen in the grafts of Walker tumor. It is also very likely that the degree of response to drug action is an inherent property of a particular cell type, and does not necessarily depend on the length of mitotic cycle or mitotic stage.

Quantatitive Analysis of the Cellular Effects

Cytologic studies have disclosed that mitotic injury induced by the various aminostilbenes in the cells of Walker carcinoma is the initial stage of growth-inhibition. It is thought, therefore, that mitotic poisoning may be a simpler and more sensitive criterion of anti-tumor activity of a chemical agent than inhibition of growth used by Haddow *et al.* (*1*). The latter effect has been found to be the combined result of various and closely integrated events, each requiring the fulfillment of specific conditions. It was, therefore, decided to restrict the analysis of the effects induced in the Walker tumor by the various aminostilbene derivatives to

the mitotic injuries only, and to use them as the criteria by which the biological activity may be related to molecular structure. It is necessary to mention that only certain aspects of the mitotic poisoning were selected for analysis and the investigation has been greatly limited in its scope by the lack of the substances for testing. The data obtained, however, are sufficient to confirm that the anti-tumor activity of the aminostilbenes is influenced by various features of their molecular configuration.

The Time of Onset of Mitotic Disturbances

The time of the earliest tumor sample in which at least 5 percent of the dividing tumor cells show the primary and complex, or secondary, cytotoxic injuries was taken to be the time of onset of these abnormalities. The data of table 3 were obtained by injecting 80 mg. per kg. of each substance intraperitoneally into tumor-bearing animals. The dose was chosen to avoid the development of systemic toxic effects in the treated rats.

TABLE 3.—*The time of onset of primary and secondary cytotoxic effects after different aminostilbenes*

Substances*	Time of onset (*0 hour is time of injection*)	
	Primary cytotoxic	Secondary cytotoxic
4-Aminostilbene (=A.S.)	10	30
4-Dimethyl A.S	12	48
2′-Chloro-4-dimethyl A.S	18	56
2′-Methyl- " "	12	48
3′-Chloro- " "	18	56–58
3′-Methyl- " "	12	48
4′-Chloro- " "	Not observed	48 (9%)†
4′-Methyl- " "	Not observed	48 (11%)†
4′-Fluoro- " "	8–10(?)	16–18
4′-Bromo- " "	10–12‡	22

*Dose was the same in all cases: 80 mg./kg.
†The incidence of cells with cytotoxic effect is shown in parentheses.
‡Data of one experiment.

It was found that the 4′-fluoro and bromo derivatives produce mitotic abnormalities about 10 hours after administration, while this interval is 18 hours when chlorine is substituted in the 2′ or 3′ position. The onset of primary effects could not be identified after the administration of 4′-chloro- and 4′-methyl-4-dimethylaminostilbene, because the incidence of abnormally dividing tumor cells did not rise above the level observed in nontreated tumors.

The time interval between the appearance of the primary and secondary cytotoxic effects varies between 6 and 40 hours. A late onset of the primary effects is usually followed by a late onset of the secondary cytotoxic injuries. The shortest interval was found after the administration of 4′-fluoro- and the longest after that of 2′- or 3′-chloro-4-dimethyl-aminostilbenes.

The effect of dosage was studied on the derivatives that had the shortest and longest time of onset. The data are shown in table 4. It seems that the primary cytotoxic effects appear earlier after the larger doses. If the mitotic disturbances are taken as a proof that the drug entered the cell, the data obtained may be interpreted as showing that 4-aminostilbene and the 4′-fluoro-substituted derivatives are much more rapidly absorbed than either the methyl- or chloro-substituted aminostilbenes. The time of onset of the mitotic abnormality is very likely determined by the solubility of the substance.

TABLE 4.—*The effect of various doses on the time of onset of primary cytotoxic effects*

Dose / Substance	4′-fluoro-4-dimethyl-aminostilbene	2′-chloro-4-dimethyl aminostilbene
(*mg./kg.*)	(*hours*)	(*hours*)
25	12*	18
50	10	18
125	8	18
250	8	17

*0 hour is the time of intraperitoneal injection.

The Frequency of Injured Cells

Effect of the substituent

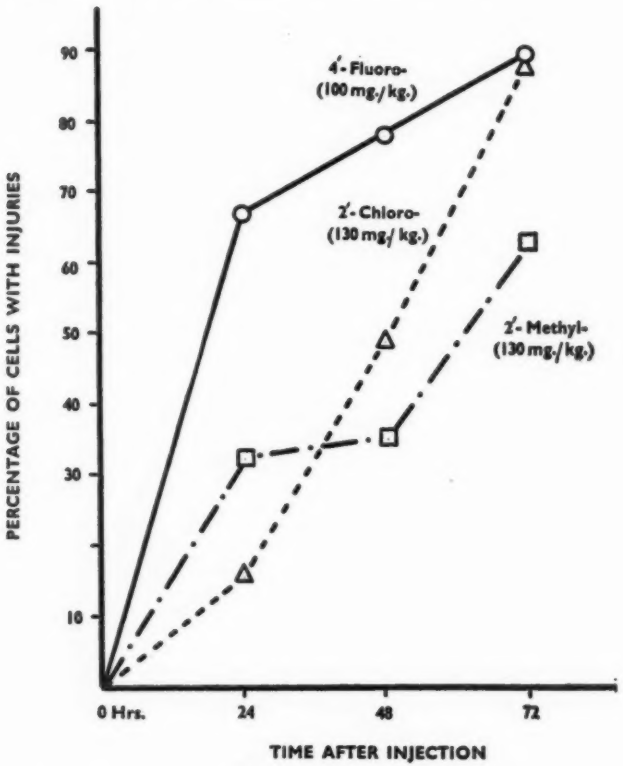
In each tumor sample taken at varying times after the intraperitoneal injection of the different aminostilbenes, 100 dividing cells were analyzed. They were in the metaphase or post-metaphase stages of mitosis. The primary cytotoxic effects have usually disappeared in the sample taken 24 and 48 hours after injection and the mitotic injuries are mostly nucleotoxic or radiomimetic effects, which can easily be identified at anaphase and telophase. More difficulty was encountered with the analysis of the cytologic abnormalities in the 72-hour-tumor samples. The mitotic disturbances were of a more complex kind and very frequently the injury was so severe that even the mitotic stage cannot be determined (*cf.* fig. 25). Furthermore, it is likely that the "abnormal" class in the 72-hour-tumor sample contains many degenerating cells in which the mitotic injuries might have been indirectly induced.

Table 5 shows the frequencies of the abnormally dividing tumor cells which appeared after treatment with various aminostilbene derivatives. The dose used (100-130 mg./kg.) was found to be highly effective, inhibiting the growth of 4-day-old Walker tumors in about 75 to 80 percent of the treated animals. It can be seen that 4′-fluoro-4-dimethylaminostilbene has the highest frequency of abnormal cells in all three tumor samples after treatment. The chloro- and methyl-substituted dimethylaminostilbenes behaved differently. At 24 hours, the methyl derivatives produced more injured cells than the chloro derivatives, while 48 and 72 hours after treatment the latter is the more effective. Text-figure 1 illustrates the observed differences in the frequency of abnormal mitosis

TABLE 5.—*The number of cells with mitotic abnormalities in the Walker carcinoma after treatment with different aminostilbenes (100 dividing cells are analyzed)*

Substances	Dose* (*mg./kg.*)	Time after injection (*in hours*)		
		24	48	72
4-Aminostilbene (=A.S.)	100	20	35	71
4-Dimethyl A.S.	130	14	26	53
2′-Chloro "†	130	15	48(41)‡	88(76)
3′-Chloro "	130	17	50	61
2′-Methyl- "†	130	33	36(40)	63(59)
3′-Methyl- "	130	29	29	50
4′-Fluoro- "†	100	67§	78(70)	88(83)
Control‖	———	7	3	3

*Average weight of rats is 185 grams.
†See text-figure 1.
‡Numbers in parentheses obtained in a second set of experiments.
§Fifteen of 67 cells have shown secondary cytotoxic effects.
‖Tumor-bearing rats were injected intraperitoneally with 1 cc. of arachis oil only.



TEXT-FIGURE 1.—Graph showing the number of cells with mitotic injuries after treatment with three aminostilbene derivatives.

for 3 aminostilbene derivatives. The methyl and chloro substituents in the 2′ and 3′ positions of the molecule apparently have the same relative effect.

The findings illustrate one of the difficulties which can be encountered when the biological activity of different substances is compared. It is evident that the effects analyzed must be compared at various times after the administration of the drugs.

The data (doses: 100 and 130 mg./kg.) show that the number of abnormally dividing cells increases with time; *i.e.*, the proportion of cells with mitotic injuries is consistently highest in tumor samples fixed 72 hours after injection. All the aminostilbenes tested at these dose levels have shown the same tendency though they may differ in the rate at which mitotic injuries appear. Thus, 24 hours after injection of 4′-fluoro-4-dimethylaminostilbene 67 percent of the dividing cells were abnormal, while after the 2′-methyl or 2′-chloro derivatives the frequency was 33 and 15 percent, respectively. On the other hand, the efficiency of the last two derivatives greatly increased by 48 and 72 hours, while that of the 4′-fluoro derivative showed little change. A similar tendency was noted when fluorine was substituted in the 2′-position (dose: 165 mg./kg.). The data could be taken to indicate that the fluoro-substituted compound has been absorbed more rapidly then either the chloro- or methyl-substituted derivatives.

In one experiment the effect of concentration was investigated (table 6). Only two aminostilbenes were tested and both gave similar results: the same dose is more effective in producing mitotic injuries at high concentration than when it is given at lower concentrations.

TABLE 6.—*The effect of different concentrations of two aminostilbenes on the number of abnormally dividing cells (50 dividing cells are analyzed)*

Substances	Dose* (*mg./kg.*)	Dissolved in arachis oil (*cc.*)	Number of abnormal cells (*in hours*)	
			24	48
2′-Chloro-4-dimethylaminostilbene...	100	0. 5	21	43
		1. 0	11	19
2′-Methyl-4-dimethylaminostilbene...	100	0. 5	30	38
		1. 0	14	21

*Average weight of rats is 180 grams.

Effect of position of the substituents

The investigations of Haddow and co-workers (*1*) have indicated that the position of a substituent in the benzene ring affects the biological activity of the aminostilbene molecule. In the latter investigations the activity was measured by inhibition of growth and the dose administered was kept well below that which produces severe systemic toxic effects and kills the animals.

In the present experiments doses varying between 50 and 550 mg. per kg. of body weight were given. The administration of aminostilbenes in doses higher than 200 mg. per kg. was usually followed by systemic toxic effects from which only few animals can recover. For that reason, in this set of experiments the cytologic analysis was restricted to tumor samples that were taken 48 hours after injection of the drugs—from animals not showing the more severe symptoms of systemic poisoning. The data given in table 7 indicate that the 2′- and 3′-positions of chloro- and methyl-substituted derivatives are much more effective than the 4′-position. Thus, the dose of the 4′-methyl derivative required to produce mitotic injuries in about the same number of cells is 11 times that of 2′-methyl-4-dimethylaminostilbene. The data show that the 2′- and 3′-positions are equally effective when methyl is the substituent, while the 2′-position has a slight advantage when chloro is the substituent.

It was found that a threefold increase in the dose doubles the number of abnormal cells 48 hours after the administration of 4′-chloro and methyl derivatives. The effect of increased dose on the frequency of abnormal cells is not so great when these substituents are in the 2′- and 3′-positions.

TABLE 7.—*The number of abnormally dividing cells 48 hours after the administration of different doses of 4-dimethylaminostilbenes (100 cells are analyzed)*

Substitution	Position	Dose (*mg./kg.*)	Number of abnormal mitoses
Chloro	2′	50	44
		150	52
	3′	50	34
		150	50
	4′	50	9
		150	18
		250	32
Control*	———	———	6
Methyl	2′	50	28
		150	36
	3′	50	26
		150	29
	4′	50	10
		150	21
		250	20
		550	29
Control	———	———	2
Fluoro	4′	75	32
		125	75
	2′	40†	No inhibition of growth
		165‡	85(?)
Control	———	———	2
Bromo	4′	125	18
		150	15
	3′	180†	Inhibition of growth
	2′	200†	No " " "
Control	———	———	7

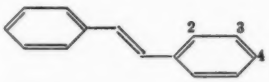
*The tumor-bearing animals were injected intraperitoneally with 1 cc. of arachis oil only.
†Data obtained by Haddow *et al.* (*1*).
‡Data of one experiment; only 35 dividing cells were seen, of which 30 were grossly abnormal (85%)!

Table 7 contains some additional data concerning two other halogen elements: bromine and fluorine. Unfortunately, these were available in sufficient quantity only in the 4′-positions and their cytologic effects could not be compared with those of the same elements in the 2′- or 3′-position. Haddow *et al.* (*1*) found a high degree of growth-inhibition after treatment with the 3′-bromo-4-dimethylaminostilbene (180 mg./kg.), and no effect after the administration of 2′-bromo-(200 mg./kg.) and 2′-fluoro-4-dimethylaminostilbene (40 mg./kg.). During the present investigation, in one experiment about 85 percent of the dividing cells showed gross injuries after treatment by 2′-fluoro derivative (dose: 165 mg./kg.). In view of these observations it may be inferred that all three halogen derivatives of 4-dimethylaminostilbenes are effective agents as mitotic poisons but their activity is influenced by the position of the substituted atom in the benzene ring.

Discussion

Activity and Molecular Structure

By comparing the growth-inhibitory proportion of a large series of stilbene derivatives, Haddow and co-workers (*1*) found that the active compounds all contained a basic substituent. The cytologic studies carried out by the present author have shown that stilbene itself is completely



inactive in producing mitotic injuries even after the administration of such doses as 200 and 300 mg. per kg. Similarly, when methyl was substituted in the 2 and 4 positions of the stilbene molecule, neither growth-inhibition nor mitotic poisoning occurred. Thus the cytologic data corroborate the conclusion arrived at by Haddow *et al.* (*loc. cit.*), according to whom the essential features of the biologically active molecule are: 1) two benzene rings, 2) the ethylenic double bond, and 3) a basic substituent.

The present studies also brought forward evidence to indicate that the position of substitution greatly influences the frequency of mitotic abnormalities. It was found that high activity is retained in compounds with chloro and methyl substituents in the 2′- or 3′-positions. Given at sublethal doses they induce mitotic injuries in the Walker carcinoma and produce a frequency of abnormally dividing tumor cells high enough to initiate the sequence of events that result in growth-inhibition. On the other hand, the sublethal doses of compounds with the same substituents in the 4′-position induce mitotic poisoning, but the incidence of the injured cells is very low and the growth of the tumor not affected. In order to increase the frequency of abnormal cells the dose of 4′-chloro, and 4′-methyl derivatives had to be increased over the lethal threshold dose. For that reason, and from the practical point of view, these derivatives can be considered to be the inactive aminostilbenes.

The question naturally arises as to the cause of the greatly reduced activity of the chloro and methyl substituents in the 4′-position. A lower solubility in a 4′-substituted derivative would naturally diminish the biological activity of the compound, and, in fact, such 4′-derivatives as have been examined (4′-chloro and 4′-methyl) are less water-soluble than the corresponding 2′-compounds (private communication: E. M. F. Roe). The fact, however, that two 4′-halogen derivatives (fluoro and bromo) do produce radiomimetic effects with high frequency, even when the dose administered is well below the lethal threshold, makes it possible that not only the physical properties but also the chemical nature of the substituted atoms in certain instances can be of primary importance.

Mode of Action

In order to analyze the complex mitotic disturbances which appear after the administration of aminostilbenes at growth-inhibiting dose levels (125–250 mg./kg.), in certain experiments, relatively much smaller doses were injected into the tumor-bearing animals. It was found that both primary and secondary cytotoxic effects were absent in the Walker carcinoma when less than 25 mg. per kg. was given. At such dose levels the various aminostilbene derivatives induced only nucleotoxic or radiomimetic chromosome injuries. About 10 percent of dividing tumor cells have fragmented chromosomes 32 hours after the injection of 20 mg. per kg. 4′-fluoro-4-dimethylaminostilbene. The incidence of abnormal cells was somewhat less (8 percent) after the same dose of the 2′-chloro derivative.

It has already been mentioned that chromosome injury of the radiomimetic type is initiated during interphase and it may be the result of a direct interference with the synthesis of chromosome material. Goldacre and co-workers (*19*) provided some evidence that certain cytologic injuries are related to disturbances in those cell functions that are primarily concerned with such synthesis. By analogy the production of nucleotoxic or radiomimetic injuries at low doses may be interpreted as a direct action by the aminostilbenes on chromosome synthesis.

It was also shown that progressive changes leading to growth-inhibition follow the radiomimetic cell injuries. These abnormalities which are complex and referred to as the secondary cytotoxic effects appear in tumor cells about 72 hours after administration. The dose producing such effects varied from 125 to 200 mg. per kg.; *i.e.*, it is about 8 to 10 times larger than the dose required for production of radiomimetic effects. It is very likely that the secondary cytotoxic injuries, such as superfragmentation of chromosomes, formation of nuclear bodies and gross abnormalities of the mitotic spindle, are manifestations of disturbances in general cell metabolism (*20, 21*).

The morphologic changes which appear in *nondividing* tumor cells support the assumption that general metabolic disturbances are in operation within the cells, which can be so drastic that the cell is killed in the

premitotic stage. In view of these findings it is very likely, therefore, that the aminostilbenes interfere with both nuclear and cytoplasmic functions of the cell and that the sphere of their action may depend on the concentration of the dose within the cell.

The cytologic abnormalities do not indicate, however, whether the drug itself or its metabolites are responsible for the gross cellular injuries. There is some evidence [*e.g.*, the influence of diet on the carcinogenic properties of some aminostilbenes (*22*); and tumor induction at particular sites, distant from the site of injection (*1*)] which suggests that metabolites of the aminostilbene may be involved in producing certain biological lesions. It is possible that such metabolites may also play a part in the production of the secondary cytotoxic injuries.

The analysis of cell reaction and mitotic injury induced in the Walker carcinoma by the 4-aminostilbenes shows that the particular molecular configuration of this group of substances has an important biological significance. The value of these chemical agents in the therapy of cancer may be increased by introducing lipophilic and hydrophilic properties into the molecule. Furthermore, the basic structure of the aminostilbenes can also serve as a model for the synthesis of other substances which perhaps will be more selective in their anti-tumor activity and less toxic in the organism than is the present series.

Summary

1) 4-Aminostilbene, 4-dimethylaminostilbene, and various derivatives of the latter were injected intraperitoneally as suspensions in arachis oil into rats grafted with Walker carcinoma 256, and their effects on tumor cells have been analyzed.

2) The mitotic disturbances induced fall into three classes: "physiological" or primary cytotoxic disturbances; nucleotoxic or radiomimetic; and complex or secondary cytotoxic effects.

3) Inhibition of growth in the Walker carcinoma was found to be due to mitotic poisoning which is accompanied by histologic changes in the tumor. The production of mitotic disturbances is only the first stage in this process.

4) The number of dividing cells showing abnormalities was observed to be the highest 72 to 96 hours after the administration of the substances at 150 to 200 mg. per kg. dose levels. This finding suggests that the onset of interphase is the most sensitive period in the mitotic cycle.

5) Malignant cells of different tumors differ in sensitivity to the same treatment. It is assumed that the degree of sensitivity is an inherent property of particular cell type, and that the response to drug action is influenced by the length and stage of the mitotic cycle.

6) The analysis of the time of onset of mitotic abnormalities and the frequency of injured cells, has shown that anti-tumor activity of the different aminostilbenes is affected by the nature and position of the substituents in the aminostilbene molecule.

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PLATES

PLATE 75

FIGURES 1 to 4.—Various stages of mitosis in the tumor cell of Walker carcinoma: 1) Metaphase (*polar view*); 2) Metaphase (*side view*); 3) Late anaphase showing the two groups of daughter chromosomes; 4) Telophase showing the constriction of the cell membrane which eventually will separate the two daughter cells. × 2,200

FIGURE 5.—Stickiness of chromosomes in metaphase—some chromosomes lie off the equatorial plate (24 hours after injection of 2′-chloro-4-dimethylaminostilbene). × 2,200

FIGURE 6.—"Pseudo-bivalent" configuration of sticky chromosomes (24 hours after injection of 3′-chloro-4-dimethylaminostilbene). × 2,200

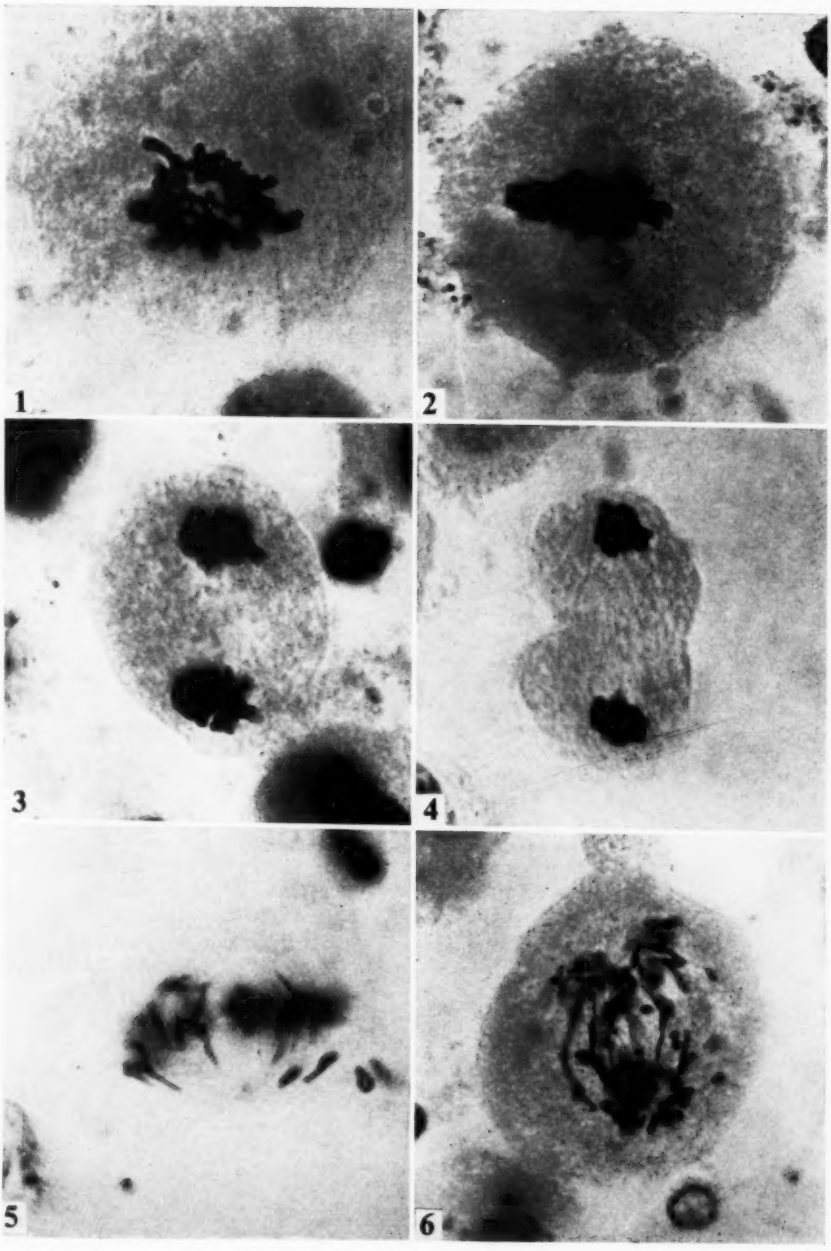

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PLATE 76

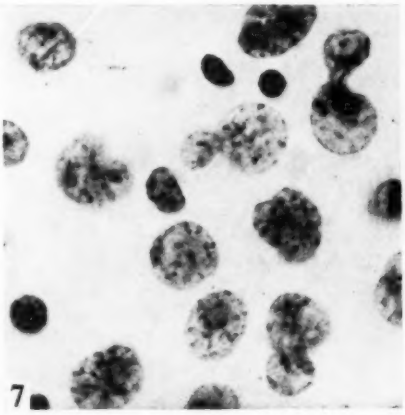
FIGURE 7.—Normal and dumbbell-shaped nuclei in tumor cells (24 hours after injection of 4′-chloro-4-dimethylaminostilbene). The small, dark-stained nuclei belong to the histiocytes. (Feulgen-stained preparation shows only the nuclei, the cytoplasm is unstained.) × 1,800

FIGURE 8.—Tumor cell in late anaphase showing several bridges which prevent the separation of the two groups of daughter chromosomes (24 hours after injection of 4′-chloro-4-dimethylaminostilbene). × 2,200

FIGURE 9.—Degenerating tumor cell with several very much condensed "nuclear bodies" (48 hours after injection of 4′-fluoro-4-dimethylaminostilbene). × 2,200

FIGURE 10.—Metaphase showing chromosome fragments (acentrics) lying off the equatorial plate (48 hours after injection of 4′-methyl-4-dimethylaminostilbene). × 2,200

FIGURES 11 and 12.—Late anaphase of tumor cells with acentric fragments. Figure 11 shows one V-shaped fragment (48 hours after injection of 2′-chloro-4-dimethylaminostilbene). In figure 12 all 5 pairs of acentric fragments joined into V-shaped structures (48 hours after injection of 3′-chloro-4-dimethylaminostilbene). × 2,200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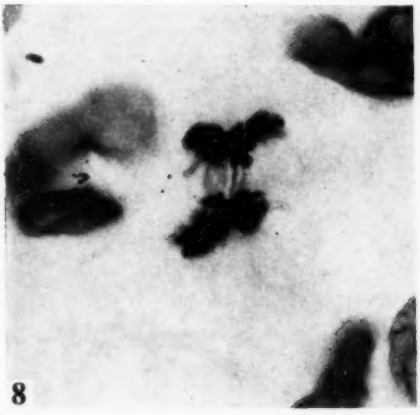
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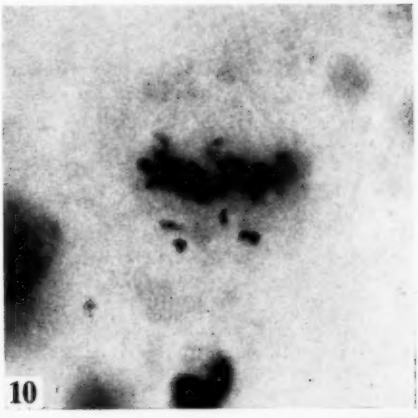
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PLATE 77

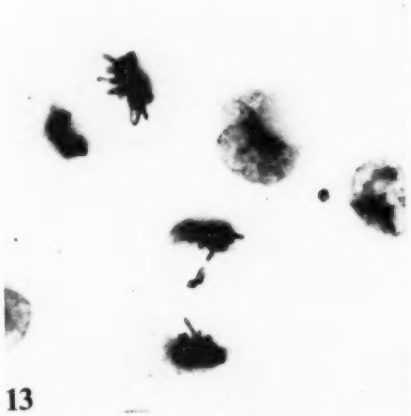
FIGURE 13.—Two dividing tumor cells in late anaphase: The one on the *left* is normal, the other has a double acentric fragment. Cell on the *right* has a small micronucleus (24 hours after injection of 4′-fluoro-4-dimethylaminostilbene). × 1,800

FIGURE 14.—Late anaphase stage of a dividing tumor cell showing ring-shaped acentric fragments (48 hours after injection of 3′-chloro-4-dimethylaminostilbene). × 2,200

FIGURE 15.—Chromosome bridges and fragments in telophase (24 hours after injection of 4′-fluoro-4-dimethylaminostilbene). × 2,200

FIGURE 16.—Two cells in "resting stage" with several micronuclei derived from acentric fragments (48 hours after injection of 3′-chloro-4-dimethylaminostilbene). × 2,200

FIGURES 17 and 18.—Abnormal mitoses of tumor cells in the presence of micronuclei that are left over from the previous division. In figure 17 the "old" micronucleus shows a partial reconstruction of the acentric fragment; figure 18 illustrates the breakdown of chromosome mechanism (72 hours after injection of 4′-methyl-4-dimethylaminostilbene). × 2,200



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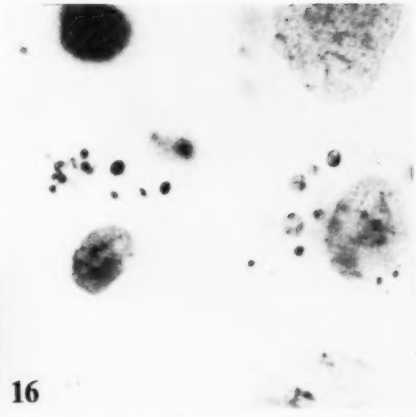

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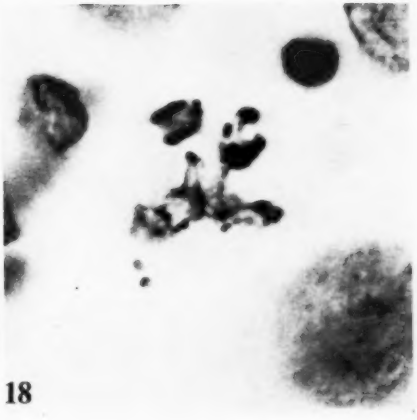

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PLATE 78

FIGURE 19.—Tumor cells in "resting stage." In the top *left* corner is a cell with a dumbbell-shaped nucleus and a large micronucleus. Two other cells contain one small micronucleus (24 hours after injection of 4′-fluoro-4-dimethylaminostilbene). × 1,800

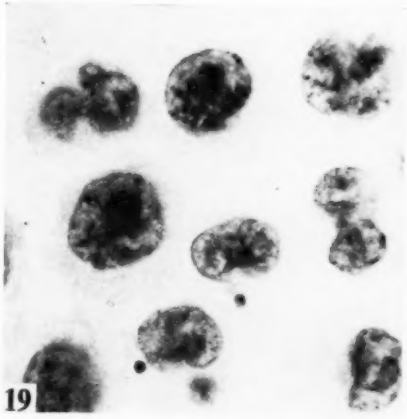
FIGURE 20.—Tumor cell in metaphase showing the long, thin, chromosomes arranged in a "hollow" spindle, which occupies the entire cell (72 hours after injection of 4′-methyl-4-dimethylaminostilbene). × 2,200

FIGURE 21.—Two dividing tumor cells: The one on the *left* is a normal telophase, the other contains short, bulky chromosomes due to supercontraction; the spindle is absent (72 hours after injection of 4′-methyl-4-dimethylaminostilbene). × 1,800

FIGURE 22.—Two abnormal mitoses: On the *left* is a cell with "hollow" spindle; the cell on the *right* contains numerous chromosome fragments. Two other cells, both in "resting stage," have a micronucleus (48 hours after injection of 4′-fluoro-4-dimethylaminostilbene).

FIGURE 23.—Dividing tumor cell with superfragmented chromosomes; the mitotic stage cannot be identified (72 hours after injection of 2′-chloro-4-dimethylaminostilbene). × 2,200

FIGURE 24.—Degenerating cell with several "nuclear bodies" that have been formed from chromosome debris. The cell on the *right* contains a large micronucleus that shows an abnormal condensation of nuclear chromatin (48 hours after injection of 4′-fluoro-4-dimethylaminostilbene). × 2,200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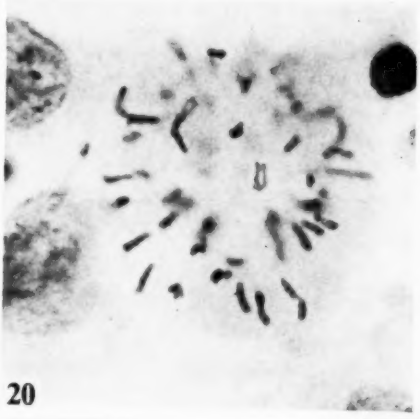
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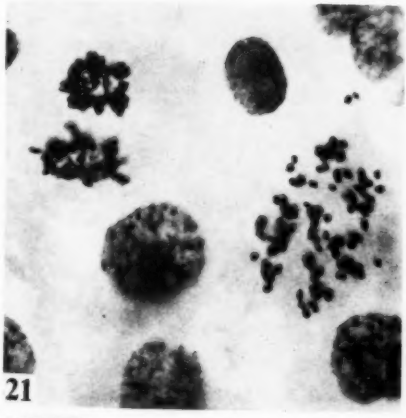
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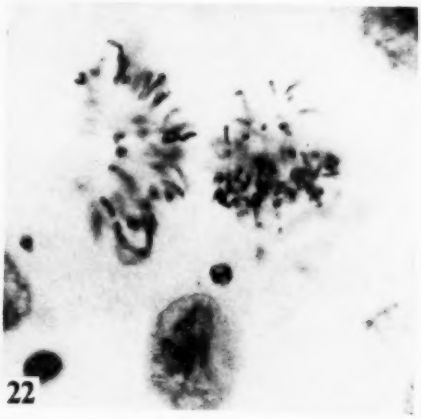
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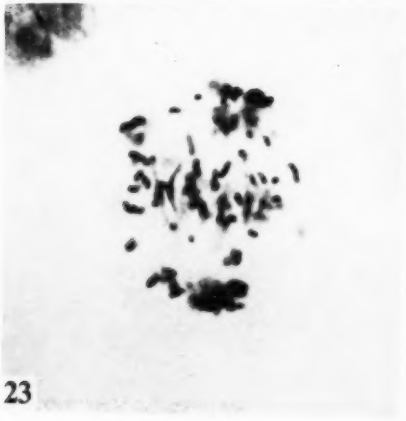
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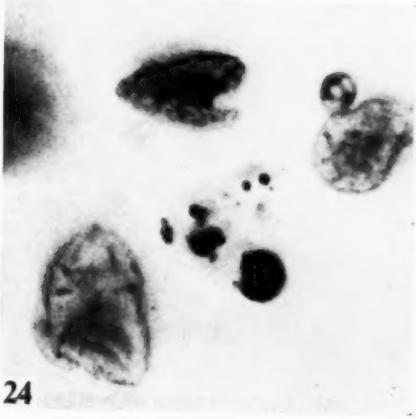
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PLATE 79

FIGURE 25.—Dividing tumor cell showing drastic damage to the chromosomes (96 hours after injection of 2′-chloro-4-dimethylaminostilbene). × 2,200

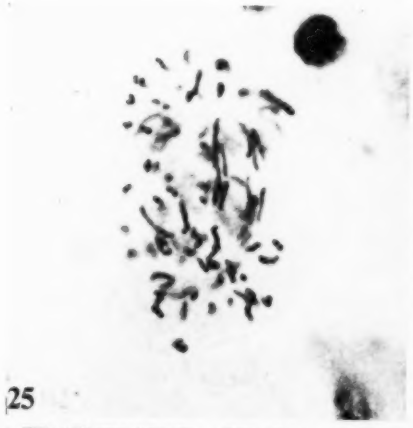
FIGURE 26.—Walker carcinoma (untreated) 8 days after grafting. It has a very cellular structure; the intercellular matrix is absent. × 320

FIGURE 27.—Walker carcinoma 6 days after treatment with 4′-fluoro-4-dimethylaminostilbene showing a great concentration of the small histiocytes, some of which are in mitosis. Only two tumor cells are present, both in resting stage. × 320

FIGURE 28.—Two dividing cells in a Walker tumor: On the *left* is a histiocyte in telophase; on the *right* is a tumor cell in late prophase. × 2,000

FIGURE 29.—Walker carcinoma showing the histologic change 8 days after the injection of 2′-chloro derivative (*cf.* fig. 26). × 320

FIGURE 30.—Abnormally enlarged nuclei in tumor cells which are embedded in a rich collagenous matrix produced by the histiocytes 8 days after the administration of 4′-fluoro-4-dimethylaminostilbene. × 320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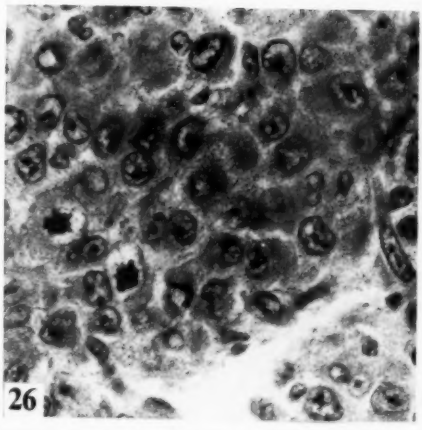
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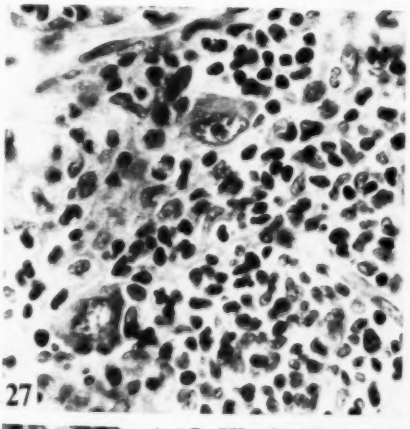
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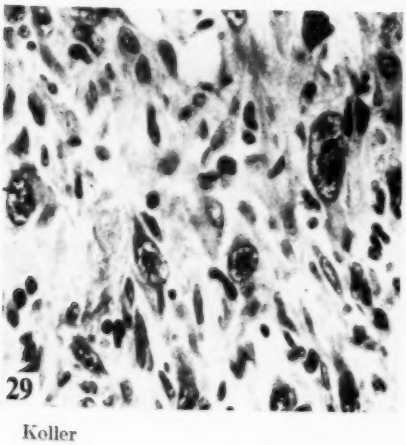
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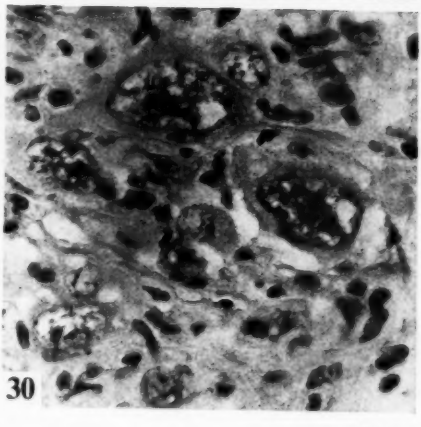
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The Chromosomes and Transplantability of Tumors. I. Fundamental Chromosome Numbers and Strain Specificity in Ascites Tumors[1]

LEO SACHS *and* RUTH GALLILY,[2] *Department of Experimental Biology, Weizmann Institute of Science, Rehovoth, Israel*

As the result of work initiated nearly 50 years ago it has been shown that the successful transplantation of a tumor is dependent on genetic factors (reviews in *1–5*). The genes concerned in the success of this transplantation are called histocompatibility (*H*) genes, because they probably also determine the success in transplantation of nonmalignant tissues (*3*). In the mouse, Snell and his co-workers have shown that *H* genes exist at different genetic loci, and that the *H-2* locus, which has been most intensively studied, exists as a series of alleles (*3*, *5*, *6*).

The genetic basis of tumor transplantation is most readily analyzed in strain-specific tumors, since these tumors normally do not grow at all, or regress, when transplanted into animals that do not belong to the strain of origin. The analysis of these tumors has shown that, in common with nonmalignant tissues, their successful transplantation requires the presence of the same *H* genes both in the tumor and in the host animal. But there are also tumors that can grow and kill when transplanted into animals which do not belong to the strain of origin, and that are therefore not strain-specific. In contrast to the strain-specific tumors and to nonmalignant tissues, tumors that are not strain-specific do not, therefore, necessarily require, for successful transplantation, the same *H* genes in the tumor and in the host animal. In view of this special property of tumors that are not strain-specific, a genetic analysis of the differences between these and the strain-specific tumors is essential for a general understanding of the genetic basis of transplantation.

The usual method of attacking this problem has been by testing the transplantability of tumors in animals belonging to the strain of origin and in animals belonging to other strains. Such transplantability tests will identify a tumor as strain-specific or as not strain-specific. But such tests alone have not been able so far to explain the genetic basis of the differences in transplantability between these two types of tumors. There is, however, another approach to this problem—the study of the chromo-

[1] Received for publication August 3, 1954.

[2] We are very much indebted to Dr. I. Berenblum for his constant interest during the course of this work. We would also like to thank Dr. G. Klein for supplying us with the ascites tumors.

somes—which has been generally used as an approach to other genetic problems. That this approach may also help to explain the genetic differences between the two types of tumors, can be seen from the results of Hauschka and Levan (*7*), who, by the comparison of different tumors, were able to find a relationship between strain specificity and chromosome ploidy. Their results show that a more complete analysis of the genetic basis of transplantation may be obtained by correlating transplantability tests for strain specificity with determinations of chromosome numbers. The object of the present investigations has been to try to obtain this more complete analysis of the genetic basis of transplantation.

In studies on the relationship between chromosome numbers and strain specificity, it is, of course, necessary to know whether the chromosome numbers of a tumor can be maintained during growth. Although the maintenance of these numbers has been inferred (*7*), the only previous systematic determinations of chromosome numbers during growth were some determinations on the Yoshida ascites tumor of the rat (*8*). In order to establish whether the chromosome numbers of tumors which we studied can be maintained under different conditions of growth, we have therefore included in the present investigation, determinations of chromosome numbers in ascites tumors of the mouse, both during growth in conditions which are immunologically compatible with the development of the tumor and also during growth in conditions which are immunologically incompatible with the development of the tumor. The genetic difference between tumors that are strain-specific and tumors that are not strain-specific has been analyzed in the present investigation by a comparison of chromosome numbers and strain specificity in five ascites tumors of the mouse.

Materials and Methods

The mouse tumors used in the present study were the ascites forms of the 6C3HED lymphosarcoma, MC1A and MC1M sarcomas, and Ehrlich and Krebs-2 carcinomas, produced by Dr. G. Klein (*9*), who supplied us with the original fluid. In order to maintain the tumors, 6C3HED, MC1A, and MC1M were propagated in C3H mice; whereas Ehrlich, and Krebs 2 were propagated in Swiss mice. The C3H mice used in our experiments were derived from an inbred stock obtained from Dr. W. E. Heston in 1951, which we had mated brother $\times$ sister since then for at least eight generations. The Swiss mice were from an originally heterogeneous stock, which we had also mated brother $\times$ sister for at least eight generations. The suitability of the ascitic fluid for inoculation was controlled at nearly every transplant generation by cell counts in a hemocytometer and by Papanicolaou smears. In all our experiments involving all five tumors, the inoculum was taken for transplantation after growing in the animals for 7 or 8 days.

The chromosome counts were made on aceto-orcein squashes, and the preparations could often be improved by pretreating the cells for about 5 to 20 minutes, either with tap water (*10*), or, better still, with a hypotonic salt solution. The pretreatment with hypotonic salt solution is the

same pretreatment method as that which has been used for tissue cultures (*11*, *12*). Whenever possible, the chromosome counts were made on the day of fixation, but if this could not be done the ascitic fluid was stored in the aceto-orcein in a refrigerator at about 5° C. All chromosome counts were made on temporary aceto-orcein preparations, but some of these preparations were made permanent by freezing the slide and coverslip on dry ice or liquid air (*13*), dehydrating, and mounting in euparal.

Results

The Accuracy of the Chromosome Counts

Tumors in the ascites form are very favorable for accurate chromosome studies. But despite their superiority in this respect over tumors in the solid form, there may still be some minor errors in the determination of chromosome numbers, because even after pretreatment with a hypotonic salt solution it is sometimes difficult to differentiate with certainty each chromosome in the cell. The degree of this difficulty varies in different tumors, as it is considerably more difficult to make counts on 6C3HED than on MC1A, MC1M, Ehrlich, and Krebs 2. In order to even out such counting errors, our data on chromosome numbers are therefore given to the nearest multiple of 5. This, therefore, gives the chromosome counts a possible error of ± 2.

Another possible source of error, which can however reasonably be neglected in evaluating the present results, may be due to the fact that there are some tumors, such as MC1A and MC1M, with very small chromatin bodies, which appear to be sometimes separate and sometimes attached to large chromosomes, and which can vary in number in different cells of the same tumor (figs. 2 to 6). Owing to the difficulty of deciding whether these very small bodies are satellites, small chromosomes, or chromatin fragments produced by chromosome breakage, we have omitted them from our chromosome counts.

Since not all the cells can be counted with the same ease, even in the same preparation of the same tumor, selection of the cells which can be most easily counted can produce errors in the frequency distribution of the chromosome numbers. We have tried to reduce this error to a minimum by selecting, as far as possible, a representative sample of the cell population.

Chromosome Numbers During Growth in an Immunologically Compatible Environment

In order to determine the distribution of chromosome numbers during growth in an immunologically compatible environment, we have examined primarily (at different intervals after intraperitoneal inoculation) the chromosome numbers of the Ehrlich ascites tumor grown in Swiss mice and of the 6C3HED ascites tumor grown in C3H mice. After intraperitoneal inoculation, Ehrlich killed 100 percent in our Swiss strain and 6C3HED killed 100 percent in our C3H strain. As it is more difficult to make

chromosome counts on 6C3HED than on Ehrlich, our chromosome counts on 6C3HED are based on samples of 25 cells, whereas our chromosome counts on Ehrlich are based on samples of 50 cells.

The Ehrlich ascites tumor.—The chromosome numbers found in the Ehrlich ascites tumor after inoculation into Swiss mice are given in tables 1 and 2. Table 1 gives the chromosome numbers at daily intervals after inoculation, as far as possible in the same transplant generation, and table 2 gives the chromosome numbers at the same time after inoculation in different transplant generations. In table 1, when cells from two generations had to be combined for the total of 50 cells per day, generations were taken which were as similar as possible in the dose and age of the inoculum, and in their chromosome distribution.

The data in table 1 show that at nearly every daily interval after inoculation, there was the same modal number, at 80, in the chromosome distribution, and the data in table 2 show that this modal number also occurs in different transplant generations. Of the 24 samples in tables 1 and 2, there were only 3 samples (days 7, 8, and 10 in table 1) which differed slightly from this general pattern. This may have been due to slight temporary shifts in the cell population, or to differences in the presence of mitoses in cells with different chromosome numbers, at the times when these samples were taken. Nearly every sample of 50 cells, therefore, showed a mode at 80 chromosomes per cell. The existence of this modal number, in the Ehrlich tumor grown in Swiss mice, is also shown by the distribution of chromosome numbers in the total of 1,200 cells which were counted in this tumor (text-fig. 1).

The Ehrlich tumor shows a considerable frequency of cells (26.6% in the total of 1,200 cells), which belong to the heterogeneous group classed as >100. In contrast to the other groups, which are all based on group intervals of only 5 chromosomes, the >100 group contains cells that range from 103 up to several hundred chromosomes. The high polyploid cells almost invariably show mitotic abnormalities, so that if these high numbers are to maintain themselves in the cell population, they will have to be continuously produced from the lower chromosome numbers by mechanisms such as endomitosis and/or a colchicine type of mitosis. This tumor, therefore, shows a considerable production of high polyploid cells, probably by these mechanisms.

The inoculation doses which were used in these chromosome determinations in the Ehrlich tumor, varied from 9 to 16×10^6 cells (mean dose $=13 \times 10^6$) for the data in table 1, and from 9 to 28×10^6 cells (mean dose$=17 \times 10^6$) for the data in table 2. There was, within this range, no consistent relationship between the number of cells used for inoculation and the frequencies of the different chromosome numbers. Thus in table 2, day 4, the inoculation dose was 11×10^6 cells for generation 47, and 27×10^6 cells for generation 52; the higher dose giving a lower percentage of cells at 80 (16% in contrast to 64%), and a higher percentage of cells at >100 (36% in contrast to 10%). However, on day 6 (table 2), the inoculation dose was 11 to 13×10^6 cells for generations 45 and

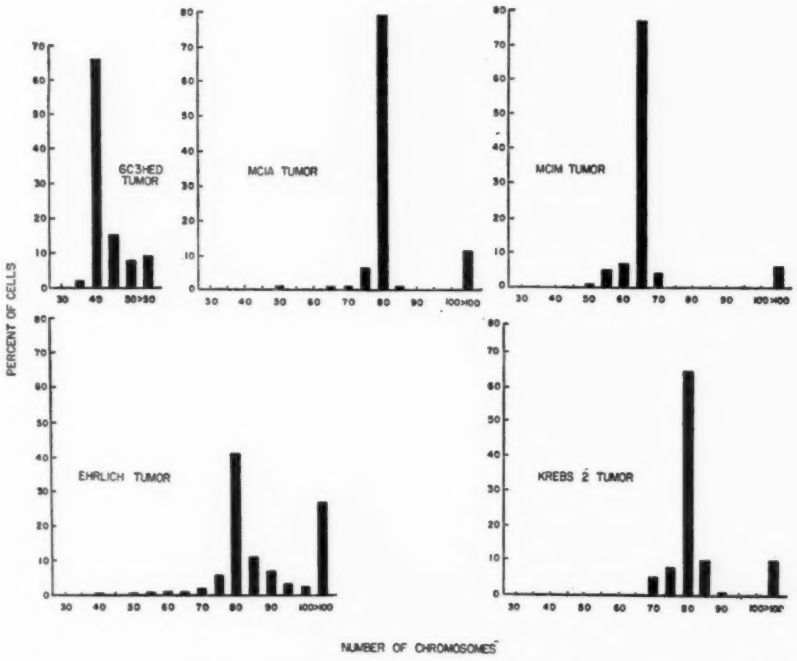
TABLE 1.—*Ehrlich ascites tumor inoculated into Swiss mice. Chromosome numbers at daily intervals after inoculation*

No. of days after inoculation	No. of transplant generation	No. of animals	No. of cells	Chromosome numbers													
				40	45	50	55	60	65	70	75	*80*	85	90	95	100	>100
1	45, 55	2	50			1	1	2		2	2	*17*	3	5	5		12
2	45, 47	2	50			1		1		2	2	*12*	5	9	1		17
3	45, 50	2	50			1					8	*27*	8		1		5
4	47	1	50	3		2	2	3			2	*8*	5	5	1	1	18
5	45	1	50	1					1	3	12	*18*	2	6	1		6
6	45, 47	2	50					1		1	3	*19*	11	6	4		5
7	45	1	50				1	2		3	2	*8*	8	10	4	6	6
8	45, 44	2	50						2	1	2	*10*	4	8	10	4	9
9	45, 47	2	50			1	1		2	1	2	*12*	11	5	1	1	13
10	45, 53	2	50							1		*14*	16	6		1	12
11	48, 53	2	50								4	*21*	5	2			18
12	48	1	50					1		1	1	*23*	4	3	1	1	15
13	45	1	50							1	5	*11*	9	6	3	1	14
Total		21	650	4		6	5	10	5	16	45	*200*	91	71	32	15	150
Total (percent)				0.6	0.0	0.9	0.8	1.5	0.8	2.5	6.9	*30.8*	14.0	10.9	4.9	2.3	23.1

TABLE 2.—*Ehrlich ascites tumor inoculated into Swiss mice. Chromosome numbers in different transplant generations on the same day after inoculation*

No. of days after inoculation	No. of transplant generation	No. of animals	No. of cells	Chromosome numbers													
				40	45	50	55	60	65	70	75	*80*	85	90	95	100	>100
1*	45, 55	2	50			1	1	2		2	2	*17*	3	5	5		12
1	54	1	50								1	*11*	1				37
1	55	1	50				1		1		1	*20*	1				26
3*	45, 50	2	50			1					8	*27*	8		1		5
3	54	1	50								2	*11*	5	2			30
4*	47	1	50	3		2	2	3			2	*8*	5	5	1	1	18
4	52	1	50	1							1	*32*	9	2			5
6*	45, 47	2	50					1		1	3	*19*	11	6	4		5
6	52	1	50							1	3	*33*	4	1			8
7	44	1	50						1		1	*11*	9	5	5	9	9
7*	45	1	50				1	2		3	2	*8*	8	10	4	6	6
10*	45, 53	2	50							1		*14*	16	6		1	12
10	48	1	50				1	1		2	1	*35*	7				3
10	54	1	50				1			1		*25*	1			1	21
10	56	1	50								2	*36*	1				11
10	56	1	50							1	1	*35*	1		1		11
10	56	1	50								7	*35*					8
Total		21	850	4		4	7	9	2	12	37	*377*	90	42	21	18	227
Total (percent)				0.5	0.0	0.5	0.8	1.0	0.2	1.4	4.4	*44.3*	0.6	5.0	2.5	2.1	26.7

*Taken from table 1.



TEXT-FIGURE 1.—The distribution of chromosome numbers (possible error = ±2) in the 6C3HED, MC1A and MC1M ascites tumors grown in C3H mice, and of the Ehrlich and Krebs-2 ascites tumors grown in Swiss mice. 6C3HED has a mode at 40; MC1M a mode at 65; and MC1A, Krebs 2, and Ehrlich a mode at 80 chromosomes. The histograms are based on the number of chromosomes in: 100 cells for MC1A, MC1M, and Krebs 2; 350 cells for 6C3HED; and 1,200 cells for Ehrlich.

47, and 27 × 10^6 cells for generation 52, and in this case the higher dose gave a higher percentage of cells at 80 (66% in contrast to 38%, or if one combines cells at 80 and at 85, 74% in contrast to 60%), and a lower percentage of cells at >100 (10% in contrast to 16%).

The 6C3HED ascites tumor.—An analysis similar to that made on the Ehrlich tumor has been made on the 6C3HED tumor, and the results are given in tables 3 and 4. The range of chromosome numbers during growth of the tumor is much lower in 6C3HED than in Ehrlich, and 6C3HED has a mode at 40 chromosomes in each one of the 14 samples examined. This modal number in 6C3HED grown in C3H mice is also shown in the distribution of chromosome numbers in the total of 350 cells which were counted in this tumor (text-fig. 1).

The inoculation doses used in the chromosome determinations in 6C3HED were 20 × 10^6 cells for the data in table 3, and 20 to 54 × 10^6 cells (mean dose = 37 × 10^6) for the data in table 4. There appeared to be, within this range, no influence of dosage on the distribution of chromo-

TABLE 3.—*6C3HED ascites tumor inoculated into C3H mice. Chromosome numbers at daily intervals after inoculation**

No. of days after inoculation	No. of transplant generation	No. of animals	No. of cells	Chromosome numbers				
				35	*40*	45	50	>50
3	48	1	25		*16*	5	1	3
4	48	1	25		*20*	4	1	
5	48	1	25	2	*15*	4	2	2
6	48	1	25		*14*	4	3	4
7	48	1	25		*16*	4	2	3
8	48	1	25	2	*14*	4	2	3
9	48	1	25	2	*14*	4	2	3
Total		7	175	6	*109*	29	13	18
Total (percent)				3.4	*62.3*	16.6	7.4	10.3

*No mitoses on 1st and 2nd day after inoculation.

TABLE 4.—*6C3HED ascites tumor inoculated into C3H mice. Chromosome numbers in different transplant generations on the same day after inoculation*

No. of days after inoculation	No. of transplant generation	No. of animals	No. of cells	Chromosome numbers				
				35	*40*	45	50	>50
6*	48	1	25		*14*	4	3	4
6	57	1	25		*18*	4	1	2
7*	48	1	25		*16*	4	2	3
7	57	1	25		*17*	3	3	2
7	60	1	25		*16*	3	4	2
7	60	1	25		*18*	3	2	2
7	60	1	25	1	*16*	5	2	1
8*	48	1	25	2	*14*	4	2	3
8	57	1	25		*20*	2	1	2
9*	48	1	25	2	*14*	4	2	3
9	57	1	25		*17*	4		4
Total		11	275	5	*180*	40	22	28
Total (percent)				1.8	*65.5*	14.5	8.0	10.2

*Taken from table 3.

some numbers. Thus, the inoculation dose was 20×10^6 cells for generation 48, 32×10^6 cells for generation 60, and 54×10^6 cells for generation 57, and day 7 (table 4) showed a similar distribution of chromosome numbers for all the three doses.

The MC1A, MC1M, and Krebs-2 ascites tumors.—In addition to the more complete analysis of chromosome numbers in Ehrlich and 6C3HED,

the chromosomes were counted in 100 cells of MC1A and MC1M grown in C3H mice, and in 100 cells of Krebs 2 grown in Swiss mice. After intraperitoneal inoculation, MC1A and MC1M killed 100 percent in our C3H, and Krebs 2 killed 100 percent in our Swiss. The chromosome counts, which were made on day 7 in MC1M, day 7 and day 9 in Krebs 2, and day 8 in MC1A, are shown in text-figure 1. Each tumor shows a mode, MC1A and Krebs 2 at 80, and MC1M at 65 chromosomes. The doses used for inoculation were 10×10^6 cells for MC1M, 13 to 16×10^6 cells (mean dose $= 14 \times 10^6$) for Krebs 2, and 20×10^6 cells for MC1A.

The modal chromosome numbers that we have found in these five ascites tumors are similar to those found in other determinations on these tumors (*14*), although in our material the modes are more pronounced in the MC1M and Krebs-2 tumors. Photomicrographs of the chromosomes in these five tumors are shown in figures 1 to 12. In each of the five tumors, the modal chromosome numbers were found in cells capable of normal mitosis.

Chromosome Numbers During Growth in an Immunologically Incompatible Environment

For the analysis of chromosome numbers during growth in an immunologically incompatible environment, we have examined the Ehrlich ascites tumor after intraperitoneal inoculation into albino rats, and the 6C3HED tumor after intraperitoneal inoculation into Swiss mice. In both cases there was only a temporary growth of the tumor, which was invariably followed by regression.

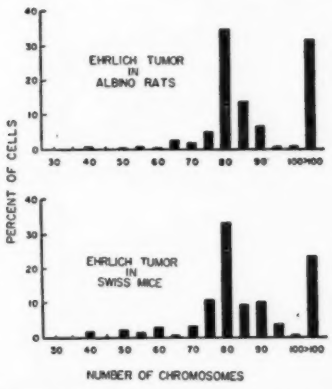
The Ehrlich ascites tumor.—The data on chromosome numbers of the Ehrlich ascites tumor grown in rats, after an inoculation of 9×10^6 cells, are given in table 5. The comparison between growth in rats and in Swiss mice, based on the days after inoculation which are the same in both cases (days 1–5 in tables 1 and 5), is shown in text-figure 2. Each sample taken during growth in the incompatible environment of the rat shows the same mode, at 80 chromosomes, as growth in the compatible environment of Swiss mice. Growth in the rat also shows the high frequency of cells in the heterogeneous>100 group, which had been found during growth in Swiss mice.

The 6C3HED ascites tumor.—The chromosome numbers of the 6C3HED ascites tumor grown in Swiss mice, after an inoculation of 27 to 34×10^6 cells, are given in tables 6 and 7 (mean dose$=28 \times 10^6$ for table 6, and 30×10^6 for table 7), and the comparison between growth in C3H and in Swiss, based on the days after inoculation which are the same in both cases (days 3–8 in tables 3 and 6), is shown in text-figure 3. In this tumor each sample also shows the same mode, at 40 chromosomes, when grown either in a compatible or an incompatible environment.

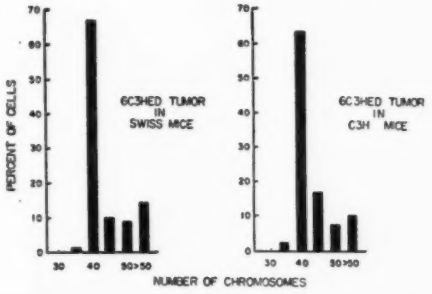
Both in Ehrlich and in 6C3HED the modal chromosome number can thus maintain itself under the conditions of growth in a host in which the tumor invariably regressed.

TABLE 5.—*Ehrlich ascites tumor inoculated into albino rats. Chromosome numbers at daily intervals after inoculation*

No. of days after inoculation	No. of transplant generation taken from Swiss mice	No. of animals	No. of cells	Chromosome numbers													
				40	45	50	55	60	65	70	75	*80*	85	90	95	100	>100
1	47	1	50							1	3	*17*	9	4	1	1	14
2	47	1	50	1						2		*16*	5	5	1		19
3	47	1	50	1		1	1	1	3		3	*22*	3	2			14
4	47	1	50							2	5	*18*	6	2			17
5	47	1	50				1		3		2	*13*	11	4		1	15
Total		5	250	2		1	2	1	6	5	13	*86*	34	17	2	2	79
Total (percent)				0.8	0.0	0.4	0.8	0.4	2.4	2.0	5.2	*34.4*	13.6	6.8	0.8	0.8	31.6



TEXT-FIGURE 2.—The distribution of chromosome numbers (possible error = ± 2) in the Ehrlich ascites tumor, after intraperitoneal inoculation into albino rats, and into Swiss mice. The tumor kills 100 percent after this inoculation into Swiss mice, but it invariably regresses after the same inoculation into albino rats. The tumor has the same mode under both conditions of growth. Each histogram is based on the number of chromosomes in 250 cells. In both cases this is a total of the samples taken from the 1st to the 5th day after inoculation.



TEXT-FIGURE 3.—The distribution of chromosome numbers (possible error = ± 2) in the 6C3HED ascites tumor, after intraperitoneal inoculation into C3H mice, and into Swiss mice. The tumor kills 100 percent after this inoculation into C3H mice, but it invariably regresses after the same inoculation into Swiss mice. The tumor has the same mode under both conditions of growth. Each histogram is based on the number of chromosomes in 150 cells. In both cases this is a total of the samples taken from the 3rd to the 8th day after inoculation.

Chromosome Numbers and Transplantability

The transplantability of the tumors whose chromosomes we had examined, was tested both by intraperitoneal and by intramuscular inoculation into C3H and Swiss mice (table 8), these being the two strains which we had available. Our results, like those of other transplantability tests on these and other tumors (7), show a relationship between the modal chromosome number and tumor transplantability. That it is the mode and not the range of chromosome numbers which

TABLE 6.—*6C3HED ascites tumor inoculated into Swiss mice. Chromosome numbers at daily intervals after inoculation*

No. of days after inoculation	No. of transplant generation taken from C3H mice	No. of animals	No. of cells	Chromosome numbers				
				35	*40*	45	50	>50
1	57	1	25		*13*	5	2	5
2	57	1	25		*12*	5	3	5
3	57	1	25		*13*	6	1	5
4	57	1	25	1	*19*	1		4
5	57	1	25		*17*	2	5	1
6	58	1	25		*12*	3	4	6
7	58	1	25		*19*	2	2	2
8	58	1	25	1	*20*	1		3
Total		8	200	2	*125*	25	17	31
Total (percent)				1.0	*62.5*	12.5	8.5	15.5

TABLE 7.—*6C3HED ascites tumor inoculated into Swiss mice. Chromosome numbers in different transplant generations on the same day after inoculation*

No. of days after inoculation	No. of transplant generation taken from C3H mice	No. of animals	No. of cells	Chromosome numbers				
				35	*40*	45	50	>50
1	50	1	25	1	*18*	4	1	1
1*	57	1	25		*13*	5	2	5
2	50	1	25		*13*	4	1	7
2*	57	1	25		*12*	5	3	5
3	50	1	25		*14*	3	2	6
3*	57	1	25		*13*	6	1	5
4*	57	1	25	1	*19*	1		4
4	58	1	25	1	*20*	1	1	2
5*	57	1	25		*17*	2	5	1
5	58	1	25		*15*	4	2	4
Total		10	250	3	*154*	35	18	40
Total (percent)				1.2	*61.6*	14.0	7.2	16.0

*Taken from table 6.

shows this relationship, can be seen from a comparison between MC1A and MC1M. Both these tumors have a range from 50 to more than 100 chromosomes, but they differed both in their mode and in their degree of transplantability.

The MC1M tumor with a mode at 65 has a transplantability which is lower than that of tumors with a mode at 80 and higher than that of the tumor with a mode at 40 (see also 7). Both the 6C3HED and MC1M

TABLE 8.—*The transplantability of five ascites tumors into C3H and Swiss mice*

Tumor	Origin of tumor	Chromosome No.	Strain of mice tested	Intraperitoneal inoculation				Intramuscular inoculation			
				Inoculum (10^6 cells)		No. of animals*	No. killed (*percent*)	Inoculum (10^6 cells)		No. of animals*	No. killed (*percent*)
				Range	Mean			Range	Mean		
6C3HED.....	*C3H*	*40*±2	*C3H*	11–50	33	250/250	*100*	23	23	20/20	*100*
			Swiss	13–47	31	0/30	*0*	23	23	0/20	*0*
MC1M.....	*C3H*	*65*±2	*C3H*	6–23	11	150/150	*100*	10–11	11	25/25	*100*
			Swiss	6–18	11	150/150	*100*	10–11	11	0/30	*0*
MC1A.....	*C3H*	*80*±2	*C3H*	15–33	23	125/125	*100*	9–14	12	30/30	*100*
			Swiss	7–27	15	125/125	*100*	9–14	12	25/25	*100*
Krebs 2.....	*noninbred*	*80*±2	*C3H*	8–18	14	20/20	*100*	8–13	10	20/20	*100*
			Swiss	4–21	12	200/200	*100*	4–13	8	20/20	*100*
Ehrlich.....	*noninbred*	*80*±2	*C3H*	7–19	15	20/20	*100*	5–20	13	20/20	*100*
			Swiss	5–21	8	250/250	*100*	5–28	17	20/20	*100*

*The first number shows the number of animals killed by the tumor, and the second number shows the number of animals inoculated with the tumor.

Animals were classed as unsuccessful transplants after an observation period of at least 3 months. In all transplants, a progressive growth of the tumor, which killed the animal, was the criterion for successful transplantation.

tumors originated in C3H mice, but 6C3HED, with a 40 mode, did not kill any of our Swiss mice either after intraperitoneal or intramuscular inoculation, whereas MC1M, with a 65 mode killed 100 percent of our Swiss mice after intraperitoneal inoculation but did not kill any of these mice after intramuscular inoculation. On the other hand the tumors with 80 modes, including MC1A, which also originated in C3H, all killed 100 percent of our Swiss mice both after intraperitoneal and after intramuscular inoculation.

In our transplantation experiments (table 8), the same results were obtained with all the inoculation doses that were used.

Discussion

The Fundamental Chromosome Numbers of Tumors

In normal somatic mammalian tissues most of the cells have the same chromosome numbers (*15–17*), and except for some tissues with polyploidy (*16, 18*), there are at most only a small percentage of cells with chromosome numbers which differ from the diploid number. For tumors it has long been known that there can be a great variation in chromosome numbers, both as regards polyploidy and as regards aneuploidy (*4, 8, 19*). But since there are also tumors in which the variation in chromosome number is no larger than that which can be found in normal tissues (*20*), the existence of great variations in chromosome numbers is not a characteristic of malignant tissues.

The data on ascites tumors show that, in these tumors with great variation in chromosome numbers, there are modes in the chromosome distribution which can be maintained during growth through many transplant generations. This can be seen both from the chromosome counts, which are dependent on the presence of mitosis, and from measurements of the amount of desoxyribose nucleic acid (*4, 9, 21*), which are not dependent on the presence of mitoses. Our analysis of the Ehrlich and 6C3HED ascites tumors has shown that the same modal number can be maintained during growth in a host which is killed by the tumor, and during growth in a host in which the tumor regresses. The stability of these modal numbers is also shown by the similarity between our chromosome counts and other chromosome counts (*14*) on the same tumors, which we examined at different times and under different conditions. The modal chromosome numbers have all been found in cells capable of normal mitosis.

The existence of a mode in all ascites tumors that have so far been examined shows that each of these tumors has, within the limits of the counting errors, a predominant chromosome number in cells capable of normal mitosis. This number can be considered as the fundamental number; and the cells, the fundamental cells of the tumor[3] (see also *8, 14, 22*). According to this concept, the genetic behavior of a tumor would be determined by the genetic constitution of the fundamental cells. That

[3] We have adopted the term fundamental cells instead of stem cells or stem line (*14*), since the latter terms already have a different well-established meaning in histology.

this supposition is correct is shown by the transplantability results, which show that differences in the fundamental chromosome number (and thus differences in the genetic constitution of the assumed fundamental cells), are correlated with differences in the genetic behavior of the tumor. It should, however, be pointed out that the concept of fundamental chromosome numbers does not imply that the same fundamental number is invariably maintained during the growth of a tumor. Whereas some tumors have the same fundamental number during many transplant generations, evidence presented in another paper (*20*) shows that during growth there can be changes in fundamental chromosome numbers that can be correlated with changes in genetic behavior.

Makino (*8*) and Makino and Kanô (*22*) have reported that the fundamental cells in several ascites tumors of the rat differ in the shape of some of their chromosomes from the chromosomes of normal tissues. Both our own and other observations (*14*) show that there are also in the mouse some ascites tumors, such as MC1M, whose fundamental cells differ in the shape of some of their chromosomes from the chromosomes of normal tissues. But in a survey of solid spontaneous carcinomas and chemically induced sarcomas we have found tumors which show no such differences from normal tissues (*20*), and among the ascites tumors this also applies to the fundamental cells of the 6C3HED tumor. The existence of such obvious differences in the shape of the chromosomes is, therefore, not a characteristic difference between normal and malignant tissues.

The Determination of Strain Specificity in Ascites Tumors

The strain specificity of a tumor is determined by its transplantability into animals belonging to strains other than the strain of origin. For the ascites tumors we have used both intraperitoneal and intramuscular inoculation for the determination of strain specificity, and our results show that in the MC1M ascites tumor, these two sites of inoculation gave different degrees of transplantability. This tumor originated in C3H mice, and both intraperitoneal and intramuscular inoculation killed 100 percent in the strain of origin. However, when this tumor was tested in Swiss mice, it killed 100 percent after intraperitoneal inoculation, but did not kill at all after intramuscular inoculation.

Hauschka (*23*) has found that a DBA tumor, inoculated subcutaneously in the solid form, gave a lower transplantability than the same tumor inoculated intraperitoneally in the ascites form. This case, however, differs from ours in that in Hauschka's (*23*) case there is a possibility of differences in the genetic constitution of the tumor cells, since Klein (*24*) has shown that the conversion of solid into ascites tumors is connected with a selection of certain types of cells. But in the experiments of Hauschka (*23*) this possibility can be tested, and in our experiments, in which we have always used the ascitic fluid, this possibility of differences in the genetic constitution of the tumor cells can be excluded.

The explanation of different *H* gene requirements for successful transplantation at different sites can probably be rejected from what is known

about tissue transplantation at different sites in the body. The difference in transplantability at the different sites of inoculation—like the difference shown between tissue grafts into the cornea (*25*), or into the anterior chamber of the eye (*26*)—compared to tissue grafts into a skin position, is probably due to differences in the effectiveness with which the immunological response can be brought to bear on the growth of the tumor at the different sites of inoculation. In support of this we have obtained evidence (Sachs and Gallily, unpublished data) by the use of ascites tumors which killed 100 percent after intraperitoneal inoculation, but which gave a lower percentage of kills or did not kill at all after intramuscular or subcutaneous inoculation. Our results show that intramuscular or subcutaneous inoculation of an ascites tumor which did not kill the mice at these sites of inoculation can modify to a varying degree, depending on the strain specificity of the tumor, the proportions of kills by subsequent intraperitoneal inoculation. It thus appears that the difference in transplantability at the different sites in the body reflects differences in the effectiveness of the immunological response. The use of both intraperitoneal and intramuscular inoculation, therefore, gives a more complete measure of strain specificity than previous determinations on ascites tumors that have been based only on intraperitoneal inoculation.

Fundamental Chromosome Numbers and Strain Specificity

A comparison of the chromosome numbers [4] and strain specificity of the five ascites tumors that we have studied shows, like the results of Hauschka and Levan (*7*), that a relationship can be found between fundamental chromosome numbers and strain specificity. Three of the five ascites tumors, 6C3HED, MC1A and MC1M, originated in C3H mice, but they differed both in their fundamental chromosome number and in their degree of transplantability. When transplanted into Swiss mice, 6C3HED with a fundamental number of 40 did not kill, either after intraperitoneal or intramuscular inoculation; MC1M with a fundamental number of 65 killed 100 percent after intraperitoneal inoculation but did not kill after intramuscular inoculation; and MC1A with a fundamental number of 80 killed 100 percent after both intraperitoneal and intramuscular inoculation. The increase in fundamental chromosome number was therefore correlated with an increase in transplantability.

That the correlation is between fundamental chromosome numbers and strain specificity and not between the range of chromosome numbers and strain specificity, can be seen from a comparison between the MC1A and the MC1M tumors. Both these tumors have the same range of chromosome numbers, but they differ both in their fundamental number and in their degree of transplantability. The production of one or a few polyploid cells in tumors with diploid fundamental cells will, therefore, not change the genetic behavior of a tumor unless these cells can eventually establish themselves as fundamental cells.

[4] As pointed out on page 1269, our chromosome counts have a possible error of ± 2.

The fundamental numbers of 40, 65, and 80, which have been found in these ascites tumors, represent different degrees of duplication of 40, the normal diploid number of the mouse. This series of numbers is very close to a polyploid series of 40, 60, 80. The relationship between fundamental chromosome numbers and strain specificity, is, therefore, a relationship between the duplicated chromosomes contained in the fundamental number, and the degree of strain specificity. By testing the transplantability of other tumors within the range of 40 to 80, including such tumors as the lines of the Ehrlich ascites tumor with a fundamental number of about 45 (*27*), it may, in fact, be possible to determine which and how many chromosomes must be duplicated for the establishment of various degrees of strain specificity. Thus if, as indicated from some of the literature, the 45 chromosome lines of Ehrlich are not strain-specific, this would show that the duplication of a relatively small amount of the right chromosome material is sufficient for the establishment of this nonspecificity.

Since tumors with a chromosome number of near 80 probably originally had a duplication of all the 40 chromosomes, whereas tumors with lower chromosome numbers probably had a duplication of only some of the 40 chromosomes, the near 80 chromosome tumors have a greater probability of containing the right duplicate chromosome material for the establishment of nonspecificity.

The duplication of chromosomes brings with it the duplication of genetic loci. By correlating transplantation tests for strain specificity with determinations of chromosome numbers, it has therefore been possible to show that the special property of successful transplantation into animals with different genotypes is related to the duplication of genetic loci. Although duplication by itself, without other changes, does not seem to be sufficient to produce a loss of strain specificity, studies on newly produced tumors have further shown (*20*), that the presence of duplication is necessary for the successful transplantation of a tumor into animals with different genotypes.

Summary

Chromosome numbers and strain specificity have been studied in five ascites tumors of the mouse.

These tumors all have a range of chromosome numbers. But among this range there is in each tumor, within the limits of counting errors, one number which is predominant in cells capable of normal mitosis. It has been shown that this number can maintain its predominance both during growth in an immunologically compatible environment, and during growth in an immunologically incompatible environment. This is therefore regarded as the fundamental chromosome number of the tumor.

Transplantability tests, which show that differences in the fundamental chromosome number are correlated with differences in strain specificity, have demonstrated that it is the cells with the fundamental number which determine the genetic behavior of a tumor.

The five ascites tumors have a fundamental number of either 40 ± 2,

65 ± 2, or 80 ± 2. This represents a difference in the number of duplicated chromosomes. A comparison of the transplantability of these tumors has shown that in these tumors the higher number of duplicated chromosomes is related to a higher degree of nonspecificity. Since duplication of chromosomes includes duplication of genetic loci, the correlation of transplantation tests for strain specificity with an analysis of the chromosomes, has therefore shown that the successful transplantation of a tumor into animals with different genotypes is related to the duplication of genetic loci.

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PLATE 80

Photomicrographs of the chromosomes of mouse ascites tumors. All × 1,500. All aceto-orecin squashes. Figures 2 and 3, pretreatment with hypotonic salt solution, and figures 4, 5, and 6, pretreatment with water.

FIGURE 1.—6C3HED tumor. Cell with 41 chromosomes.

FIGURE 2.—MC1M tumor. Cell with 61 chromosomes (+ small chromatin bodies).

FIGURE 3.—MC1M tumor. Cell with 65 chromosomes (+ small chromatin bodies).

FIGURE 4.—MC1A tumor. Cell with 78 chromosomes.

FIGURE 5.—MC1A tumor. Cell with 79 chromosomes (+ small chromatin bodies).

FIGURE 6.—MC1A tumor. Cell with 77 chromosomes.

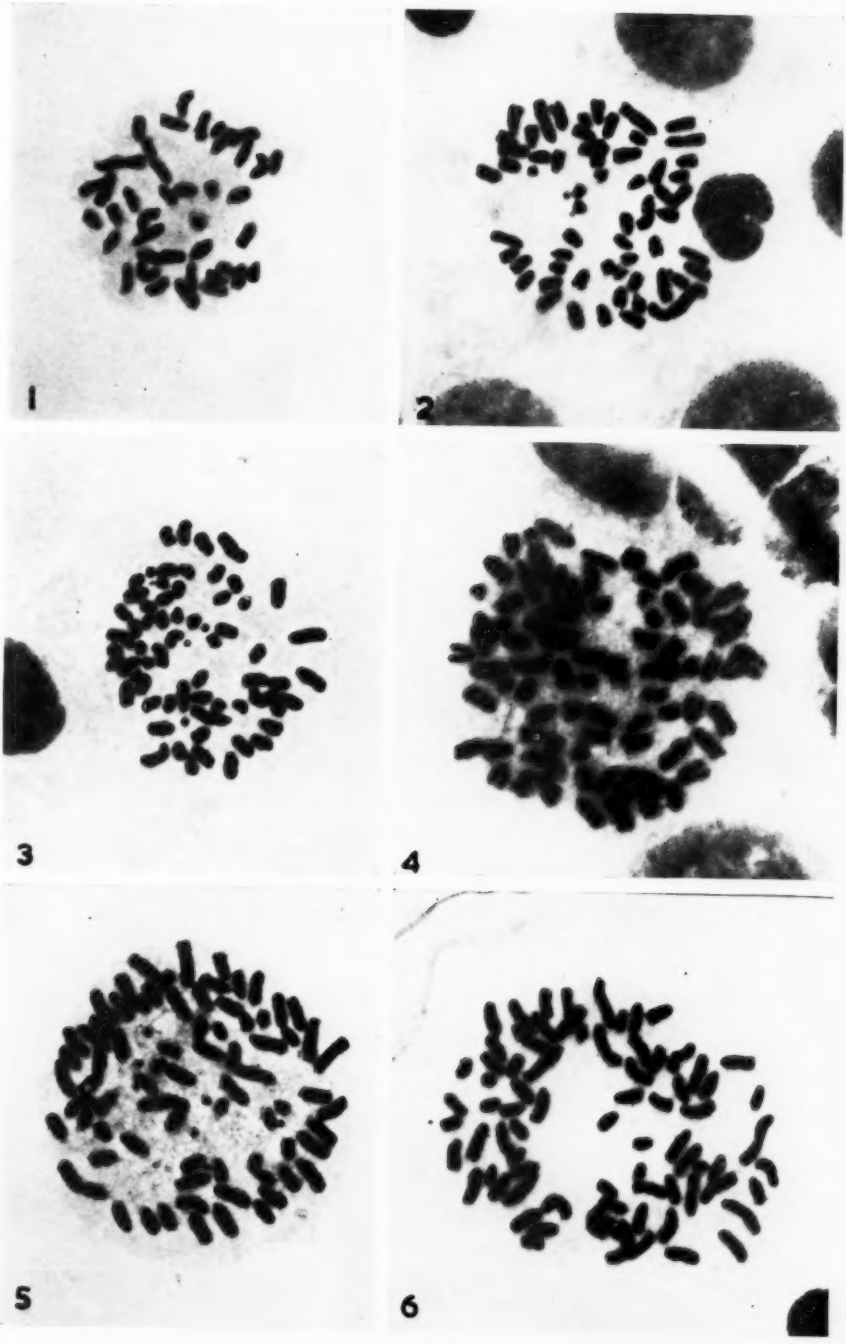

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PLATE 81

Photomicrographs of the chromosomes of mouse ascites tumors. All × 1,500. All aceto-orecin squashes. Figures 7, 9, 11, and 12, pretreatment with hypotonic salt solution.

FIGURE 7.—Ehrlich tumor. Cell with 79 chromosomes.

FIGURE 8.—Ehrlich tumor. Cell with 64 chromosomes.

FIGURE 9.—Ehrlich tumor. Cell with 158 chromosomes.

FIGURE 10.—Ehrlich tumor. Cell with 52 chromosomes.

FIGURE 11.—Krebs-2 tumor. Cell with 81 chromosomes.

FIGURE 12.—Krebs-2 tumor. Cell with 79 chromosomes.

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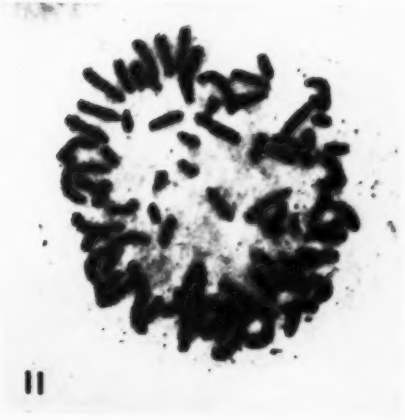
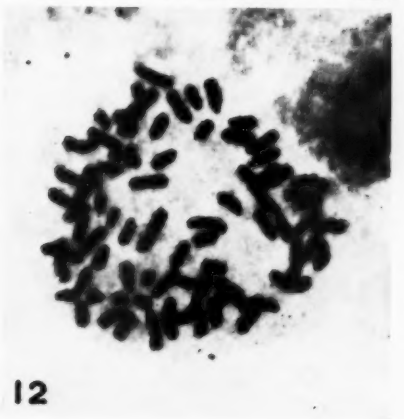


11



12

Technique for the Induction of Metastasizing and Transplantable Fibrosarcomas With Croton Oil[1, 2]

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Several recent publications from this Institute have reported the relationship between the actions of adaptive hormones (ACTH, STH, corticoids) upon inflammation on the one hand and upon neoplasms on the other (*1–3*). These investigations were guided by the working hypothesis that even the growth of cancers might perhaps be influenced at will through hormones which govern the development of their connections with the host. Although the malignant tumor cells themselves are largely autonomous, they nevertheless depend upon a connective-tissue stroma for their architectural organization and upon a blood supply for their nutrition. Both of these structures come from the host and are subject to regulation by the host's hormones (*4*).

This view appears to have received some experimental support from recent observations showing that the development of various transplantable malignant tumors of the rat and mouse can be inhibited through treatment with excessive doses of an antiphlogistic hormone, such as cortisol[4] or cortisone, and that this inhibition can be nullified in turn by concurrent administration of a prophlogistic hormone, such as STH (*6*).

The question then arose as to the possible role of inflammation (and of the hormones regulating inflammation) in the actual induction of malignant tumors. The literature concerning the effect of nonspecific irritation and inflammation upon carcinogenesis is voluminous, but rather contradictory (*7, 8*). Recently, however, it became possible to adapt the "granulomapouch" technique—originally designed for the quantitative study of inflammation caused by chemical irritants, microbes or tumor transplants (*1, 9, 10*)—so as to render it especially suitable for the re-examination of this topic. With this new procedure, extreme concentrations of strong irritants can be applied to a closed connective-tissue

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[2] This investigation has been performed with the aid of the National Cancer Institute of Canada.

[3] The author is also greatly indebted to Mr. K. Nielsen, who prepared the photomicrographs, and to Miss Odette Treich for technical assistance.

[4] In agreement with the suggestion made by Shoppee (*5*), the term cortisol is used here in preference to "hydrocortisone," since it eliminates confusion with 4,5-dihydrocortisone and obviates the possible implication that "hydrocortisone," is to be regarded primarily as a mere derivative of cortisone.

sac for periods of several months without the occurrence of either local necrosis and perforation to the outside, or noteworthy systemic intoxication.

In the present communication, we should like to describe the results of the first experiments in which this technique was used. They show that by merely placing 100-percent croton oil in constant contact with connective tissue during several months it is possible to produce a very high percentage of extremely malignant, metastasizing and readily transplantable fibrosarcomas in the rat.

Materials and Methods

Experimental animals.—For the induction of the tumors 12 female albino Sprague-Dawley rats, weighing 130 to 155 grams, were used. The resulting tumors were then transplanted into female Sprague-Dawley or Wistar rats of the same body-weight range.

All experimental animals were fed exclusively on "Purina Fox Chow." In those used for the induction of tumors, great care was taken to prevent as far as possible any accidental injury to the skin in the granuloma pouch area. For this purpose, the fur on the back was repeatedly shaved to facilitate periodic washing and cleansing of the skin surface. Since rats tend to scratch the skin which covers the granuloma pouch, we trimmed the points of their claws from time to time to prevent self-inflicted injuries. These precautionary measures greatly diminish the number of failures in experiments designed to maintain granuloma pouches over long periods of time. Normally necrosis and perforation tend to occur at points where dermal lesions, due to parasites or scratch injuries, induce surface inflammation with eventual adherence of the skin to the granuloma pouch wall. Whenever the separation from the skin by loose connective tissue is thus interrupted, perforation usually follows.

Even with all these precautions, 5 of our 12 rats had to be eliminated in the course of the first 200 days of observation because of incidental skin injuries due to infection from the outside following scratches that may have been inflicted by rubbing against the metallic cage walls or food containers.

Production of the "inexhaustible" granuloma pouch.—In the 12 rats that were to be used for the induction of tumors, a granuloma pouch was prepared on the *1st day* of the experiment in the following manner:

First, 25 ml. of air were insufflated under the shaved dorsal skin through a #27-gauge, subcutaneous injection needle. Then, after detaching the syringe without withdrawing the needle, 0.5 ml. of a 0.5-percent solution of croton oil (U. S. P.) in corn oil was injected into the air sac so formed. This resulted in the usual granulomatous transformation of the air-sac wall, with the consequent production of hemorrhagic exudate.

On the *4th day*, just when exudation began to take place, the air was removed through a #27 needle. Numerous preliminary experiments had shown that such a collapse of the pouch helps to accomplish a clear separation of the granulomatous sac from the skin through a layer of loose con-

nective tissue. It had been noted that as a consequence of this separation, even if subsequently strong irritants are injected into the pouch cavity, its wall does not tend to undergo the usual phenomenon of "local tissue exhaustion"; that is, progressive inflammatory infiltration of the adjacent skin and finally necrosis conducive to the evacuation of the pouch contents.

After the pouches had thus been rendered highly resistant, a second stronger dose of 0.5 ml. of 20-percent croton oil was injected into the exudate-containing cavity on the *10th day*. This was rapidly followed by considerable distension of the granuloma pouches by large amounts of liquid, but even during this period of intense exudation the granulomatous walls did not become adherent to the cutaneous covering, and the skin could easily be moved about over the granuloma surface.

On the *30th day*, the whole accumulated exudate (10 to 30 ml.) was removed. We had to do this with a thick #18-gauge needle, since by that time numerous clots had formed in the cavity. If we had inserted this needle through the "roof" of the pouch, it would have produced a puncture large enough to be incapable of spontaneous watertight closure. Hence, we introduced the needle through a rather tortuous and long connective-tissue channel underneath the back of the skin, behind the pouch, entering the latter through its "floor" (that is, the surface adjacent to the muscle and not the skin). Even so, we did not venture to inject strong croton oil into the pouch immediately after evacuation, but first merely filled its cavity with an amount of air which approximately corresponded to the volume of exudate withdrawn. Only after 24 hours on the *31st day*, when the puncture hole was presumed to be more solidly closed by a clot, did we proceed with the next injection of 0.5 ml., now of 100-percent croton oil.

Essentially the same procedure was repeated on the *103rd day*, when the newly formed exudate was again evacuated from all pouches (15 to 35 ml.) and replaced with air. This was followed on the *104th day* by the injection of 1 ml. of 100-percent croton oil into the air-space.

The animals were then left untouched for several months, the first of them being killed on the *217th day* for histologic study and transplantation.

This somewhat complicated procedure of preparation may be summarized as follows:

1st day: injection of 25 ml. of air +0.5 ml. of 0.5% croton oil;
4th day: evacuation of air (to induce delimitation of granuloma from adjacent skin);
10th day: injection of 0.5 ml. of 20% croton oil into exudate-containing, granuloma pouch cavity;
30th day: evacuation of exudate from granuloma pouch, replacing it by equal volume of air;
31st day: injection of 0.5 ml. of 100% croton oil into air-space;
103rd day: evacuation of newly accumulated exudate, replacing it by equal volume of air;
104th day: injection of 1 ml. of 100% croton oil into air-space;
217th day: sacrifice of first tumor-bearing rat.

Technique of transplantation.—The first two tumors that developed in the walls of the granuloma pouches were tested for transplantability. For

this purpose, specimens of neoplastic tissue were removed, under sterile conditions, immediately after killing the bearers. The skin over the tumor region was shaved and sterilized by contact with a red-hot metal spatula. An incision was then made through the burned area and specimens of the tumor removed. These were subsequently treated in a glass homogenizer, after addition of physiologic saline. In the case of the first tumor a great excess of saline was used, while the second tumor was mixed with saline, in a proportion of 1:2, so as to obtain a more concentrated suspension.

The recipient rats were all shaved in the regions selected for inoculation and their skin was cleansed with alcohol and metaphen. After this, 1 ml. of the tumor-suspension was injected at various sites, as described below. Obvious tumor nodules became visible in most cases between the third and sixth days. Their structure was histologically verified in all cases.

Observations

As mentioned above, 5 of the original 12 rats had to be eliminated at various times during the course of the experiment. However, on the 217th day, when the first tumor-bearing rat (subsequently referred to as No. 1) was sacrificed, mere inspection and palpation showed that nodular, rapidly growing neoplasms had also developed in the pouch walls of four other rats. One of these (No. 2) was killed on the 226th day, two (Nos. 3 and 4) on the 228th day, while the last one (No. 5) died spontaneously on the 235th day of the experiment.

The granuloma pouches of the two remaining rats (Nos. 6 and 7) revealed no obvious indication of neoplastic transformation by the 240th day. These animals were killed at that time in the hope that histologic examination might reveal the aspect of the first stages in the sarcomatous transformation. Great cellular polymorphism and rather atypical fibroblasts and polynuclear giant cells were seen in several foci of the otherwise rather acellular, thick, fibrous pouches obtained from both of these rats. However, the changes were not sufficiently characteristic of cancer to substantiate a diagnosis of malignancy. It is undoubtedly possible that, in these two rats, the lesions might never have progressed to the point of transformation into an invasive metastasizing and transplantable neoplasm.

The *macroscopic* aspect of the neoplastic granuloma pouch of rat No. 1 is illustrated in figures 1 and 2. The tumors of the other rats were essentially similar. The amount of fluid found in the pouch cavity varied from case to case between 58 and 170 ml. It had a peculiarly viscous, soapy consistency and a bright yellow color. The weights of the dissected tumors themselves varied between 35 and 120 gm. and the final body-weights of the bearers (after removal of the tumors) between 212 and 285 gm.

In tumor No. 5, the fluid contents of the neoplastic granuloma pouch were evacuated three times at weekly intervals, prior to the animal's death, to relieve pressure. Each time, about 100 ml. had accumulated

within a week and the newly formed fluid continued to be extremely viscous and of yellow color. It is conceivable that this last animal's death was hastened as the result of these repeated drainings.

Histologically, the neoplastic granuloma pouch itself exhibited the characteristics of a more or less typical spindle-cell sarcoma (*cf.* figures 1 to 9) in all cases, although in some areas very atypical polynuclear giant cells were also quite common—for instance, in tumor No. 2. It is unlikely that this extreme polymorphism was due to the continued presence of croton oil or necrotic tissue debris, as it was also commonly seen in young metastases and transplants.

Metastatic deposits of the neoplastic granuloma pouch tissue were observed only in the lungs of rats Nos. 1 and 5. The first of these is illustrated in figures 8 and 9.

Tumors Nos. 1 and 2 were *transplanted* immediately after sacrificing the bearers. A tissue-suspension from the first was inoculated subcutaneously into five Sprague-Dawley rats but it took only in two of them, presumably because we used a very highly diluted aqueous suspension that had been homogenized a little too vigorously, so that most of the cells were presumably damaged or dead. Nevertheless, in these two animals, the tumor grew to considerable size and eventually killed the hosts.

The more concentrated cell-suspension prepared from tumor No. 2 was transplanted as follows:

1) Intramuscularly into the thigh in five Sprague-Dawley rats. Result: five takes, no regressions.

2) Intramuscularly into the thigh in five Sprague-Dawley rats treated daily with 5 mg. of cortisone subcutaneously. Result: five takes, no regressions.

3) Intramuscularly into five Wistar rats. Result: five takes, but these grew very slowly in comparison with the transplants in Sprague-Dawleys; five regressions.

4) Intraperitoneally in five Sprague-Dawley rats. Result: five takes, with diffuse peritoneal sarcomatosis and intense ascites formation. These rats were most severely damaged and began to die before all others. Three succumbed on the ninth day and, at that time, we killed the remaining two which were moribund.

5) Subcutaneously under the back, in five Sprague-Dawley rats. Result: four takes, two of which grew very slowly and one regressed.

6) Into a 25-ml. subcutaneous dorsal air-sac (prepared according to the granuloma pouch technique, but using tumor-suspension as an irritant instead of croton oil) in six Sprague-Dawley rats. Result: six takes, rapidly growing in the pouch wall and causing intense exudation; no regressions. These animals lost weight rapidly and died between the 14th and 20th day. All others (except those with intraperitoneal grafts) were still quite well at this time and died only during and after the fourth week.

Histologically, the structure of the transplanted tumors resembled that of the primary neoplasms and their metastases.

A rather surprising incidental observation in the bearers of these transplants was an often extraordinarily intense involution of the spleen and atrophy of the liver. This was unexpected because the hosts of many other transplantable rat tumors (*e.g.*, Walker tumor 256) usually exhibit a characteristic hepatomegaly and splenomegaly. The thymus was also extremely atrophic in our transplant-bearers. This is not unusual with intraperitoneal grafts of other neoplasms, although it is rarely of such intensity.

Discussion

These findings indicate that under our experimental conditions it is possible to induce, with some regularity, highly malignant connective-tissue tumors by topical treatment with croton oil in the rat.

There has been much discussion about croton oil as a cocarcinogen, but all investigators seemed satisfied that it is not a true carcinogen capable of inducing neoplasia in itself (*11–13*). Indeed, the very concept of cocarcinogenesis is based upon the assumption that certain irritants such as croton oil, "without being in themselves carcinogenic, could augment the tumor-producing action of a carcinogenic agent when the degree of irritation by the latter was insufficient" (*14*).

It may be added that many irritants, when applied repeatedly to the skin, together with a potent carcinogenic tar, allegedly even exert a pronounced inhibitory influence on tumor production (*15, 16*). Although within one large group of irritants, tested in conjunction with a carcinogenic tar, only a small number showed any pronounced anticarcinogenic action—and some even acted as cocarcinogens—they were not carcinogenic in themselves (*17*).

In an extensive series of mice treated with cutaneous applications of croton oil, one animal did develop a tumor following repeated applications of croton oil and acetone. Great importance was attached to this case, because at first sight it could have been taken as evidence that croton oil is itself at least a weak carcinogen. However, Berenblum (*14*) felt that "as against this it should be pointed out (*a*) that the 'tumor' in question was a minute pedunculated wart which regressed after a short time, never to reappear, (*b*) that an occasional small wart of this kind, which never grows or becomes malignant, is not infrequent with other apparently noncarcinogenic irritants, such as xylene, as mentioned by Kennaway (*18*), oleic acid as noted by Twort and Fulton (*19*), and even the anticarcinogen, dichloroethylsulfide" (*16*). However, Salaman and Roe (*20*) likewise induced papillomas with croton oil in mice.

Burrows (*21*) produced chronic inflammation by the subcutaneous injection of such irritants as powdered crystalline silica, bouillon or starch and then administered potent carcinogens at other sites. In a series of 362 rats and mice there did not occur a single tumor at the focus of irritation. It was concluded that "these results offer no support to the frequently repeated statement that nonspecific 'chronic irritation' is an

agent in the causation of neoplasia." On the other hand, spindle-cell sarcomas did occasionally occur in rats (though not in mice) given parenteral injections of various fats (*22*). In mice 0.5-percent HCl, buffered with phthalate, can produce sarcoma in the subcutaneous tissue when injections are continued over a relatively long period of time (*23*). Concentrated solutions of glucose and other sugars injected repeatedly into the subcutaneous tissue of rats or mice may induce sarcomas after many months (*24–26*). Sarcomas have also been induced in albino rats, following the introduction of a thick regenerated cellulose film, either by wrapping the cellophane around one kidney or by embedding it subcutaneously in the abdominal wall (*27*).

In all those experiments in which tumors had apparently been induced by inflammatory irritants it would be difficult to exclude the possibility of a contamination with traces of some highly potent carcinogens. This criticism, of course, also applies to our own work with undiluted croton oil. It is noteworthy, however, that under our experimental conditions in this Institute no tumor formation was ever observed in rats bearing ordinary granuloma pouches prepared in the routine manner with 0.5-percent croton oil in corn oil. Such pouches are invariably absorbed within a few months, without leaving any detectable trace. It is highly probable, therefore, that the consistent induction of malignant tumors with simple inflammatory irritants (*e.g.*, croton oil) is possible only if these are topically applied for a long time and in very high concentrations, such as are normally not tolerated because they induce tissue necrosis.

Under ordinary conditions, it is, of course, impossible to expose the tissues of the rat to concentrated croton oil, as they would be completely destroyed by it and then eliminated from the body. On the other hand, the lining of the "inexhaustible" granuloma pouch—prepared according to the technique described in this communication—becomes extraordinarily resistant to the necrosis, so that here undiluted croton oil can be placed in persistent contact with living rat tissues.

In *conclusion* it may be said that our technique has enabled us to apply concentrated croton oil to connective tissue under standardized and controllable conditions. It makes it possible to obtain a long-continued effect without the necessity of constantly repeated treatments and permits the application of the irritant to an artificially created, closed inner surface of any desired size, under sterile conditions. Thus the procedure avoids the danger of complicating incidental exogenous injuries and infections, as well as uncontrollable losses of the applied chemical, which make corresponding experiments on the skin so unsuitable for quantitative analysis.

With this technique it became possible to show that sarcomas can develop as a result of constant irritation with concentrated croton oil.

We have not yet compared the biology of the grafted croton-oil-induced neoplasms with other transplantable tumors of the rat. However, at least in the Sprague-Dawley strain in which our tumors have originated,

they take in virtually 100 percent of the cases and then grow rapidly until they finally kill the host.[5]

It is hoped that the technique of the granuloma pouch will also prove useful in examining the possible carcinogenic effect of various other simple inflammatory irritants, applied in otherwise not tolerable concentrations. In this sense it may serve as a basis for systematic studies on the relationship between inflammation and neoplasia. It is planned to examine, for instance, the influence upon tumors thus induced by simple irritation (though not primarily "true carcinogens") of such factors as systemic stress and the adaptive hormones (ACTH, STH, corticoids), that is, agents which are known to exert an important influence on non-neoplastic tissue reactions to local irritation, such as inflammation and necrosis.

Summary

It has been possible to modify the "granuloma pouch" technique (originally devised for the study of inflammation) so that it permits the establishment, in a circumscribed connective-tissue cavity, of such tissue resistance that even concentrated croton oil, applied constantly during a period of more than 200 days, causes no necrosis or perforation to the outside.

Among seven rats treated with 100-percent croton oil in this manner, five developed highly malignant sarcomas in the granulomatous tissue immediately surrounding the deposit of irritant.

The neoplasms so induced grow rapidly and can metastasize to the lung. They are also readily transplantable and eventually fatal to other rats of the same strain.

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[5] A supply of animals bearing such tumors is constantly maintained at our Institute and will be placed at the disposal of interested investigators upon request.

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PLATE 82

FIGURE 1.—*Macroscopic aspect of a rat bearing neoplastic granuloma pouch (after shaving the skin).*—Note the large size of the granuloma pouch and the numerous, irregular nodules of neoplastic tissue, as well as the dilated veins visible through the skin.

FIGURE 2.—*Same after the covering skin flap is dissected and turned back.*—Note the cutaneous muscle layer, whose fibers originate from the axillary region and then fan out over the entire pouch area. The dark partially hemorrhagic exudate shines through the pouch at places where the granuloma is still thin and not invaded by the tumor. The nodules of grayish-white neoplastic tissue are clearly distinguishable in this photograph. They were extremely hard and cartilaginous in consistency.

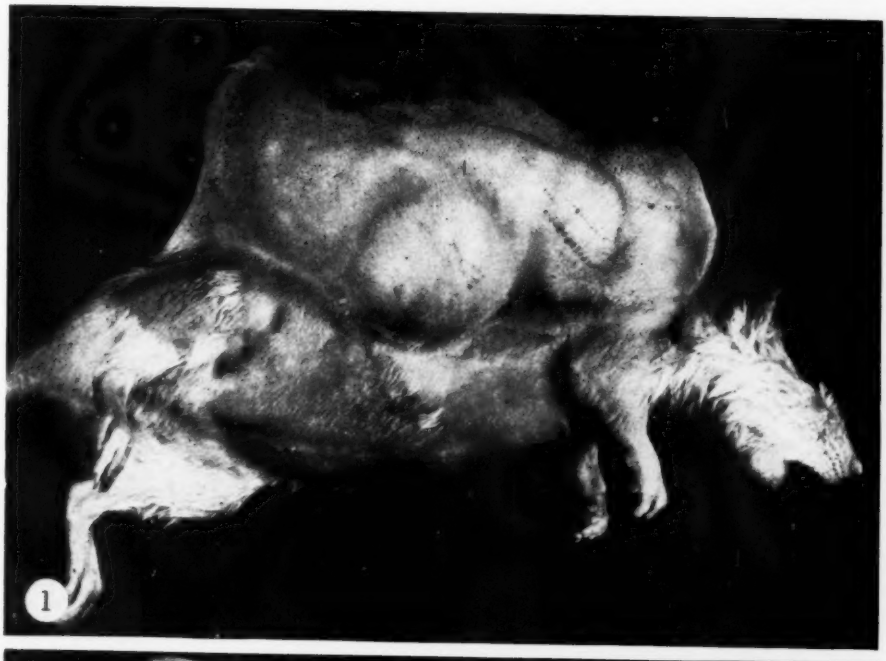

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PLATE 83

FIGURE 3.—*Inner surface of neoplastic granuloma pouch, as it appeared following evacuation of its fluid content.*—Note the numerous, apparently quite independent light gray neoplastic nodules. The greatest accumulation of cancer tissue is seen near the cranial (*top*) pole of the pouch; here the thickness is made visible by a deep incision through the width of the wall. Near the caudal pole much of the granuloma tissue is still free of cancer.

FIGURE 4.—*Section through the neoplastic granuloma pouch wall at very low magnification.*—The surrounding capsule is rich in blood vessels (*bottom*) and consists of ordinary connective tissue. The inner surface, on the other hand, is transformed into a solid neoplastic layer. × 40

FIGURE 5.—*Higher magnification of a region near the inner surface of the neoplastic granuloma pouch.*—Here the tissue consists almost exclusively of very polymorphic malignant cells. × 450

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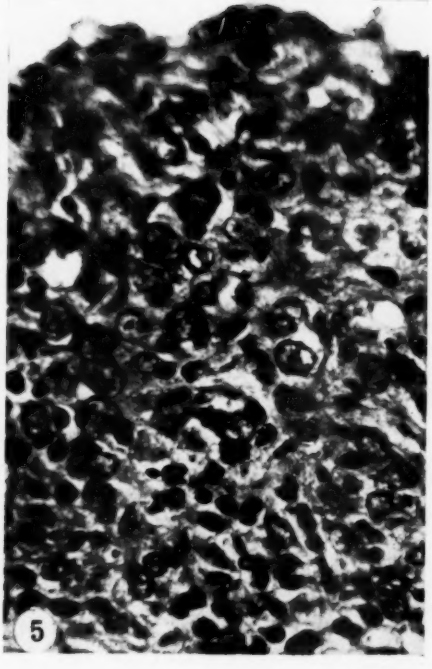

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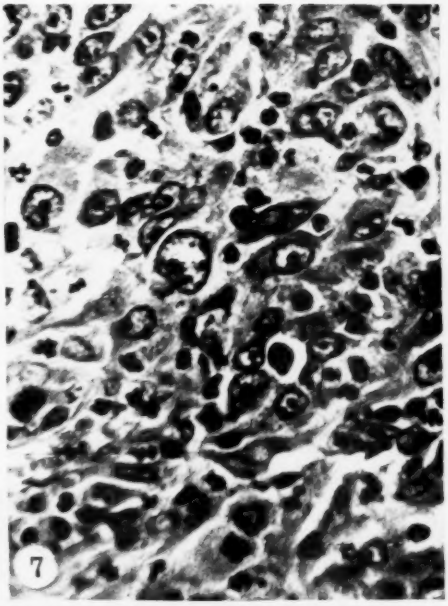
PLATE 84

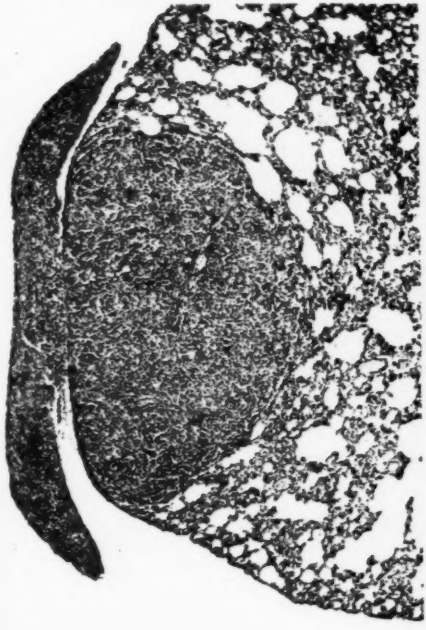
FIGURE 6.—*High magnification of an area taken from the depth of the cancerous cranial pole of the pouch.*—Note great pleomorphism of the nuclei and atypical cell divisions. The predominantly spindle-shaped character of the cells and the structure of their nuclei suggest that we are dealing with a highly malignant fibrosarcoma. × 450

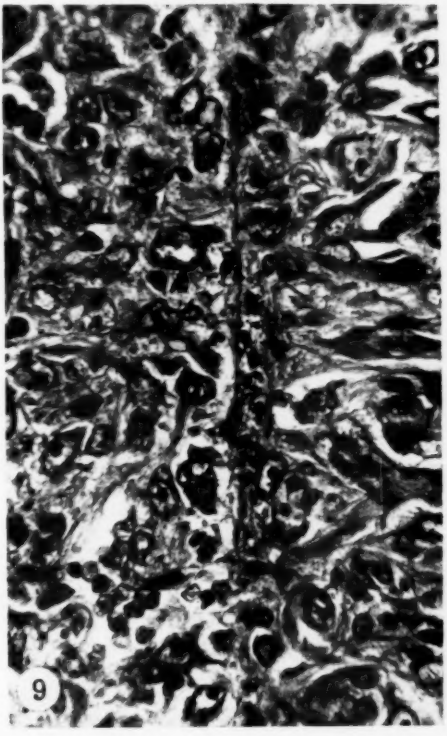
FIGURE 7.—*A still more polymorphic area from the same tumor, showing several "monster cells."*—Note also rather diffuse infiltration of the neoplastic tissue with polymorphonuclear leukocytes in this region. × 450

FIGURE 8.—*Small pulmonary metastasis of fibrosarcoma, near the apex of the right lung.*—This solid neoplastic tissue was easily distinguishable even at naked-eye inspection. Note invasion of the tumor into the pleural space in the form of a mushroomlike process that originates from a limited point of the pleura. × 40

FIGURE 9.—*High magnification of the perforation point shown in figure 8.*—Note that the original pleural surface is still clearly distinguishable as a straight line traversing this field from top to bottom. On the *right* is the intrapulmonary metastasis; on the *left* the intrapleural infiltration. Highly atypical neoplastic cells are visible on both sides of the pleural demarcation line. × 45







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Mortality Attributed to Lung Cancer in the Large Cities of the United States in 1948 and 1949[1]

ALEXANDER G. GILLIAM, M. D., *National Cancer Institute,*[2] *Bethesda, Md.*

In a previous communication (*1*) comparisons were made among the States of the United States of average annual mortality attributed to cancer of the lung for the years 1948 and 1949. Even after account was taken of differences in the age, race, sex, and residence (urban-rural) components of their populations, considerable variation in lung-cancer mortality was demonstrated among the States. The data also showed an excessive mortality rate in the urban as compared with the rural population of the entire United States.

It therefore appears desirable to examine the data for the entire urban population to determine whether there was similar variation among the cities which comprise it. Since a 2-year mortality experience is insufficient to reliably demonstrate differences that might exist between individual areas of small population size, it is not feasible with these data to examine lung-cancer mortality in the small cities. Comparisons are possible, however, for the 106 cities that in 1950 had a population of 100,000 and over, and it is the purpose of this paper to make these comparisons.

Methods

In the previous paper comparisons among States were made by use of Standardized Mortality Ratios (SMR's), which in principle are similar to the SMR's so usefully employed in publications of the Registrar General of England and Wales. As used in the previous study they represented *ratios*, adjusted by the indirect method to the specific rates for age, race, sex, and residence (urban-rural) of the entire United States, and expressed as a percentage of the United States *rate* for all persons. Since the SMR is flexible, permitting expression of adjustment for several factors in one index, and readily convertible into an adjusted rate, it will also be employed in this study.

In making these comparisons some reason might be offered for grouping the cities further into several population subclasses. There are 5 cities

[1] Received for publication September 27, 1954.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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of 1,000,000 and over, 13 of 500,000 to 1,000,000, 23 of 250,000 to 500,000, and 65 of 100,000 to 250,000. As seen in table 1 there is some tendency for rates to increase with increase in size of population. This tendency, however, is quite irregular. Among white males, for example, the difference in rates for cities of 100,000 to 250,000 and 250,000 to 500,000 is immaterial, as is the difference between the last two classes. Thus, in this race-sex group the greatest divergence in rates is between cities below and above 500,000 population. In the other three race-sex components the change in rates with size of cities follows different patterns. Because of this irregularity of the tendency for rates in the large cities to increase with increase in size of the city, it appears more reasonable to make comparisons among the 106 cities on the basis of standardization to rates for all cities in the broad class of 100,000 or more population, rather than subgroup them and compare each with the subdivision to which it belongs. This paper therefore deals with comparisons of the 106 cities with the average for all cities in the one broad class—100,000 or more population.

When average annual age, race, and sex specific mortality rates for all cities of 100,000 and over (as given in table 1) are applied to the appropriate individual city populations, expected deaths for each city are obtained. Dividing the deaths observed in each city by the deaths expected, gives a ratio which when multiplied by 100 gives the SMR for the city. The SMR as used in this study is thus a *ratio*, adjusted by the indirect method for age, race, and sex and expressed as a percentage of the *rate* for all persons living in cities of 100,000 or more population. Because this SMR is necessarily adjusted to different base rates from those used in the previous study, it obviously cannot be compared to the SMR ot that study and can be used only to compare the cities of 100,000 population and over among themselves.

Results

Table 2 shows the SMR's for cancer of the lung for the 106 cities of the United States which in 1950 had a population of 100,000 or more. In addition, for those States having more than 1 city of this size, the table shows the SMR for all population in each State residing in cities of 100,000 population and over. It is observed that the ratios vary from a low of 32 in Charlotte, N. C., to a high of 147 in Jersey City, N. J. That is to say, the rate in Charlotte, adjusted by the indirect method for age, race, and sex, is only 32 percent of the rate for all United States population residing in cities of 100,000 and over, while the rate in Jesery City is 47 percent greater than the average.

On the basis of this limited 2-year mortality experience, 5 cities (New Orleans, Baltimore, St. Louis, Jersey City, and New York City) had significantly excessive ratios (at $P = 0.01$ or less) and in 15 cities the ratios were significantly lower than the average. As shown in table 3 when the SMR's are distributed by groups of cities of different population classes, it is observed that *a*) none of the 65 cities of 100,000 to 250,000 population had significantly excessive ratios but 9 had significantly low ratios; *b*)

TABLE 2.—*Standardized mortality ratios (SMR)* for cancer of the lung in 1948–49, for the people of each State who lived in cities having a population of 100,000 or more in 1950*

State and city	Expected deaths	SMR
Alabama	132	94
Birmingham	80	81
Mobile	29	106
Montgomery	23	124
Arizona—Phoenix	36	117
Arkansas—Little Rock	30	70
California	1, 448	90†
Berkeley	36	82
Long Beach	90	86
Los Angeles	690	93
Oakland	137	85
Pasadena	44	76
Sacramento	53	74
San Diego	97	77‡
San Francisco	301	94
Colorado—Denver	138	69†
Connecticut	208	85‡
Bridgeport	54	108
Hartford	61	72‡
New Haven	56	93
Waterbury	37	59†
Delaware—Wilmington	37	126
District of Columbia	221	104
Florida	183	97
Jacksonville	53	96
Miami	89	82
Tampa	41	131‡
Georgia	109	95
Atlanta	82	88
Savannah	27	116
Illinois	1, 262	99
Chicago	1, 224	100
Peoria	38	71
Indiana	294	80†
Evansville	39	61‡
Fort Wayne	43	72
Gary	38	121
Indianapolis	136	83‡
South Bend	38	54†
Iowa—Des Moines	60	81
Kansas	87	95
Kansas City	39	112
Wichita	48	81
Kentucky—Louisville	109	89
Louisiana	208	122†
Baton Rouge	24	34†
New Orleans	153	137†
Shreveport	31	119
Maryland—Baltimore	279	116†
Massachusetts	561	95
Boston	277	107
Cambridge	37	111
Fall River	37	90
New Bedford	41	81
Somerville	34	90
Springfield	59	63†
Worcester	76	77‡
Michigan	677	88†
Detroit	568	91‡
Flint	48	78
Grand Rapids	61	68†
Minnesota	338	68†
Missouri	450	114†
Kansas City	157	106
St. Louis	293	118†
Nebraska—Omaha	85	106
New Jersey	409	118†
Camden	39	115
Elizabeth	37	94
Jersey City	97	147†
Newark	140	118‡
Paterson	52	100
Trenton	44	103
New York	3, 254	121†
Albany	51	90
Buffalo	204	105
New York City	2, 699	127†
Bronx	493	127†
Brooklyn	896	121†
Manhattan	730	144†
Queens	515	114†
Richmond	65	126‡
Rochester	131	78†
Syracuse	78	84
Utica	39	66‡
Yonkers	52	87
North Carolina—Charlotte	28	32†
Ohio	973	93‡
Akron	89	71†
Canton	41	95
Cincinnati	175	97
Cleveland	306	106
Columbus	119	79‡
Dayton	75	76‡
Toledo	111	92
Youngstown	57	91
Oklahoma	120	78‡
Oklahoma City	68	75‡
Tulsa	52	82
Oregon—Portland	150	95
Pennsylvania	1, 065	103
Allentown	39	70
Erie	43	79
Philadelphia	672	108‡
Pittsburgh	225	104
Reading	42	104
Scranton	44	80
Rhode Island—Providence	86	81
Tennessee	212	78†
Chattanooga	34	70
Knoxville	32	67
Memphis	98	80‡
Nashville	48	86
Texas	511	78†
Austin	31	51†
Corpus Christi	21	52‡
Dallas	113	77‡
El Paso	30	101
Fort Worth	75	61†
Houston	141	95
San Antonio	100	77‡
Utah—Salt Lake City	53	70‡
Virginia	113	125†
Norfolk	46	134‡
Richmond	67	119
Washington	292	76†
Seattle	178	84‡
Spokane	62	57†
Tacoma	52	71‡
Wisconsin—Milwaukee	220	80†

Jacksonville	53	96
Miami	89	82
Tampa	41	131‡
Georgia	109	95
Atlanta	82	88
Savannah	27	116
Illinois	1, 262	99
Chicago	1, 224	100
Peoria	38	71
Indiana	294	80†
Evansville	39	61‡
Fort Wayne	43	72
Gary	38	121
Indianapolis	136	83‡
South Bend	38	54†
Iowa—Des Moines	60	81
Kansas	87	95
Kansas City	39	112
Wichita	48	81
Kentucky—Louisville	109	89
Louisiana	208	122†
Baton Rouge	24	34†
New Orleans	153	137†
Shreveport	31	119
Maryland—Baltimore	279	116†
Massachusetts	561	95
Boston	277	107
Cambridge	37	111
Fall River	37	90
Canton	41	95
Cincinnati	175	97
Cleveland	306	106
Columbus	119	79‡
Dayton	75	76‡
Toledo	111	92
Youngstown	57	91
Oklahoma	120	78‡
Oklahoma City	68	75‡
Tulsa	52	82
Oregon—Portland	150	95
Pennsylvania	1, 065	103
Allentown	39	70
Erie	43	79
Philadelphia	672	108‡
Pittsburgh	225	104
Reading	42	104
Scranton	44	80
Rhode Island—Providence	86	81
Tennessee	212	78†
Chattanooga	34	70
Knoxville	32	67
Memphis	98	80‡
Nashville	48	86
Texas	511	78†
Austin	31	51†
Corpus Christi	21	52‡
Dallas	113	77‡
El Paso	30	101
Fort Worth	75	61†
Houston	141	95
San Antonio	100	77‡
Utah—Salt Lake City	53	70‡

Massachusetts	561	95
Boston	277	107
Cambridge	37	111
Fall River	37	90
New Bedford	41	81
Somerville	34	90
Springfield	59	63†
Worcester	76	77‡
Michigan	677	88†
Detroit	568	91‡
Flint	48	78
Grand Rapids	61	68†
Minnesota	338	68†
Duluth	41	41†
Minneapolis	190	57†
St. Paul	107	96
El Paso	30	101
Fort Worth	75	61†
Houston	141	95
San Antonio	100	77‡
Utah—Salt Lake City	53	70‡
Virginia	113	125†
Norfolk	46	134‡
Richmond	67	119
Washington	292	76†
Seattle	178	84‡
Spokane	62	57†
Tacoma	52	71‡
Wisconsin—Milwaukee	220	80†
All cities in U. S. with population of 100,000 and over	14, 438	100

*See text for definition and method of computing SMR.

†P=0.014 or less. (P=Probability that an equal or greater difference between state or city rate and the United States average, could arise from sampling errors.)

‡P=0.015-0.054.

TABLE 1.—*Average annual death rates (1948–49) per 1,000,000 from cancer of the lung for the United States population living in cities of 100,000 or more population*

Age	White males					Nonwhite males				
	Total all cities 100,000 and over	Cities of—				Total all cities 100,000 and over	Cities of—			
		100,000 to 250,000	250,000 to 500,000	500,000 to 1,000,000	1,000,000 and over		100,000 to 250,000	250,000 to 500,000	500,000 to 1,000,000	1,000,000 and over
0–29	2.6	1.6	1.8	3.8	3.0	4.9	3.5	2.0	5.5	6.8
30–34	19.4	16.8	17.6	20.1	21.2	28.6	11.2	24.0	16.3	46.2
35–39	48.2	44.0	35.6	70.1	45.6	68.4	44.4	57.8	79.2	77.3
40–44	139.5	143.8	131.9	167.5	127.5	171.3	152.4	154.6	187.5	177.1
45–49	325.2	288.0	320.0	349.2	333.8	265.4	210.3	271.8	298.2	266.5
50–54	594.7	551.6	540.2	624.2	622.6	558.2	428.2	502.2	570.6	649.0
55–59	968.1	857.0	869.1	1020.8	1035.4	817.0	474.0	678.1	1054.4	879.4
60–64	1241.6	1045.0	1064.7	1161.7	1448.4	859.8	735.0	795.2	986.0	872.4
65–69	1333.6	1200.0	1175.3	1433.8	1423.0	732.0	491.4	753.1	1015.8	653.4
70–74	1364.1	1019.0	1167.4	1468.0	1595.9	573.6	694.6	631.8	472.0	580.6
75 and over	1089.4	770.0	793.6	1150.0	1069.5	707.2	218.6	676.0	721.0	1111.4
Total	291.5	240.5	245.7	309.2	331.8	161.2	117.4	156.6	184.6	170.1
Age adj.*	267.5	228.4	232.8	281.1	288.0	197.1	142.2	182.8	224.7	216.8
	White females					Nonwhite females				
0–29	1.8	1.8	1.5	1.2	2.2	1.0	0.0	0.0	2.5	0.8
30–34	8.1	2.9	8.4	9.4	10.8	8.7	9.6	0.0	14.2	8.6
35–39	21.8	18.1	24.4	20.9	22.1	26.4	19.2	20.0	28.6	31.3
40–44	31.7	21.5	30.3	23.9	41.0	56.8	58.6	85.2	52.8	45.3
45–49	66.3	58.5	44.0	73.1	76.3	86.0	39.5	81.4	20.5	154.9
50–54	92.7	84.0	67.2	80.2	113.6	122.0	101.4	139.1	80.6	160.4
55–59	141.9	94.4	149.4	133.3	166.6	145.4	49.0	151.6	156.8	173.4
60–64	203.8	166.8	146.2	157.0	272.6	179.3	101.2	242.8	185.8	183.7
65–69	251.4	205.9	189.1	228.1	318.7	161.1	64.2	168.6	166.8	217.6
70–74	291.6	238.7	331.2	246.4	327.6	172.6	184.8	324.2	105.0	121.8
75 and over	366.7	316.6	386.3	316.9	417.4	164.2	227.2	119.9	49.6	242.3
Total	61.1	48.8	55.0	55.2	73.8	37.0	26.1	43.4	30.2	44.2
Age adj.*	53.6	43.5	48.2	47.3	64.8	46.5	32.7	52.9	37.8	56.4

*Adjusted to the age distribution of the entire United States population in 1950. Deaths according to International List #47b, c, d, e, (1948) and #162, 163 (1949). Population according to 1950 Census.

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of the 23 cities with 250,000 to 500,000 population the ratio in 1 was significantly high and in 4 was significantly low; *c*) among the 13 cities of 500,000 to 1,000,000 the ratio in 2 was significantly low and in 3 significantly high; and *d*) one of the 5 cities of 1,000,000 or more population had a significantly excessive ratio.

TABLE 3.—*Distribution of lung cancer SMR's for cities of 100,000 or more population*

SMR	All cities	Cities of—			
		100,000–250,000	250,000–500,000	500,000–1,000,000	1,000,000 and over
30–49	3 (*3*)*	3 (*3*)			
50–59	6 (*5*)	5 (*4*)		1 (*1*)	
60–69	7 (*4*)	5 (*2*)	2 (*2*)		
70–79	21 (*2*)	15	6 (*2*)		
80–89	20 (*1*)	11	8	1 (*1*)	
90–99	16	8	3	3	2
100–109	15	6	2	5	2
110–119	10 (*2*)	7	1	2 (*2*)	
120–129	4 (*1*)	3			1 (*1*)
130–139	3 (*1*)	2		1 (*1*)	
140–149	1 (*1*)		1 (*1*)		
Total	106 (*20*)	65 (*9*)	23 (*5*)	13 (*5*)	5 (*1*)
Number of deaths recorded, 1948 and 1949	14, 438†	2,524	2,321	3,055	6,538

*Figures in parentheses indicate number of cities with SMR significantly different from average for all cities of 100,000 and over at $P = 0.01$ or less.

†In these years an additional 9,691 lung-cancer deaths were recorded in urban places of 2,500 to 100,000 population and 9,051 deaths in rural areas. (U. S. total = 33,180.)

It thus seems clear that even on the basis of a limited mortality experience of only 2 years there is a rather marked and significant variation in reported lung-cancer mortality among some of the large cities of the United States. If the numerical variation observed in other cities, which is not significantly different from the average in a 2-year experience, should persist over a longer time period, then the variation among all the large cities would indeed be impressive. The differences observed among American cities, furthermore, are similar to those previously described for English cities by Stocks (*2*).

For the metropolitan areas of 10 of the cities listed in table 2, data on prevalence, incidence, and mortality from cancer were obtained in either 1947 or 1948 through special surveys conducted by members of the Biometry Section of the National Cancer Institute (*3*). Incidence was defined in these surveys as the annual rate of diagnosis of new cases of cancer. From data provided by the Biometry Section, Incidence Ratios have been computed for these 10 cities. The Incidence Ratio represents a *ratio*, adjusted by the indirect method for age, race, and sex, expressed as a percentage of the total lung-cancer incidence *rate* for these 10 cities combined. In table 4 the Incidence Ratios for one year (either 1947 or 1948) are compared with the SMR's for the 2-year period, 1948 and 1949.

It is observed in table 4 that in terms of lung-cancer mortality recorded during 1948 and 1949 these 10 cities combined are representative of all United States cities of 100,000 or more population since their total SMR (99) is remarkably close to the average. The table also shows that the rank order of many individual cities is not the same when the 2-year SMR is used as an index as when the 1-year Incidence Ratio is employed. It is clear, however, that whether measured by a 2-year mortality ratio or a 1-year incidence ratio there is considerable variation among the 10 cities in lung-cancer experience. It cannot be determined from these or from other presently available data whether the differences observed are due to environmental, economic, diagnostic, reporting, occupational, or other factors.

TABLE 4.—*Rank order in mortality from, and incidence of, cancer of the lung among the ten cities included in the National Cancer Institute morbidity survey*

City	SMR* for two years, 1948 and 1949	Incidence ratio† for one year, 1947 or 1948
New Orleans	137‡	137‡
Philadelphia	108§	113§
Pittsburgh	104	89
Chicago	100	94
San Francisco	94	108
Detroit	91§	108
Atlanta	88	62†
Birmingham	81	88
Dallas	77§	107
Denver	69‡	81
All ten cities	99	100
All U. S. cities of 100,000 and over	100	

*When the SMR's are computed on the basis of the total 10-city experience, the values of SMR differ from those given here but the rank order of the cities remains the same.

† See text for definitions and method of computing SMR's and Incidence Ratios.

‡ Significantly different from average at level of $P = 0.014$ or less.

§ Significantly different from average at level of $P = 0.015$–0.054.

Summary

Comparisons of mortality attributed on death certificates to cancer of the lung during a 2-year period, 1948 and 1949, have been made among the 106 United States cities of 100,000 or more population. In addition to demonstrating an irregular tendency for rates to increase with the size of the city, these comparisons show considerable variation among the cities in mortality due to this disease. The data also indicate that the combined experience of the 10 metropolitan areas included in the morbidity survey conducted by the National Cancer Institute is representative of all large cities combined.

These data provide no clue as to whether the geographic differences exhibited are due to environmental, economic, diagnostic, reporting, occupational, or other factors.

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Maintenance of Brown-Pearce Tumor Under Conditions Suitable for Chorio-allantoic Culture[1,2]

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It is well known that tumors may be maintained for long periods under suitable conditions of cold storage without complete loss of viability, so that subsequent growth occurs on transplantation into the host or in tissue culture. In this laboratory the Brown-Pearce tumor has been grown in the rabbit after storage for as long as 48 days at −10° C. (*1*). However, we have not been able to use such stored tissue for growth on the chorio-allantoic membrane (CAM) of the chick embryo. Although the tissue shows definite growth, its size increase during the permissible culture period (extending from the 8th to the 17th days of incubation) was insufficient to permit multiple subcultures needed for maintenance of the tumor in the egg. Experiments were therefore undertaken to determine storage conditions which would permit tumor transplants to attain the minimal required size (about 10 mm.^{3}).

If a tumor is to be maintained on the CAM, it is also important to know whether its character is altered as a result of prolonged culture. Armstrong and Ham (*2*), Taylor and Carmichael (*3*), and Armstrong, Gray, and Ham (*4*) have reported that certain mouse and rat tumors become toxic for the chick embryo after serial cultivation in the yolk sac. Thus Taylor and Carmichael (*3*) find it necessary to pass the C3H tumor through the mouse host after culture in 6 successive eggs in order to annul its toxic effects upon the chick. Although we are not aware of reported toxic effects of tumors grown on the CAM, it was thought desirable to make observations on the mortality of the chick hosts and on possible pathologic effects of the Brown-Pearce tumor during prolonged cultivation.

Materials and Methods

The Brown-Pearce carcinoma of the rabbit, maintained by intra-ocular and intra-mesenterial transplantation in the rabbit (Dutch breed) for the

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[2] Supported in part by Cancer Research Funds of the University of California.
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past 10 years, was used. Whitish-to-pinkish nodules were removed from the surface of the gut and omentum, cut into 1 to 2 mm. pieces, and two such pieces were placed upon the junctions of large chorio-allantoic vessels of New Hampshire eggs previously incubated 8 to 9 days at 37 or 38° C. (*5*). The opening in the shell was covered with sterilized dialysis tubing held in place by cellulose (Scotch) tape. The procedure was repeated in subsequent egg-to-egg transfers.

Tumor carried through 19 generations on the CAM was implanted into the anterior chamber of the rabbit eye following the method of Green (*6*). Tumor carried for 25 and 26 subcultures was implanted into the omentum and mesentery of the rabbit following the method of Jacobsen (*7*).

Details concerning methods of storage of the tumor are presented below.

Results

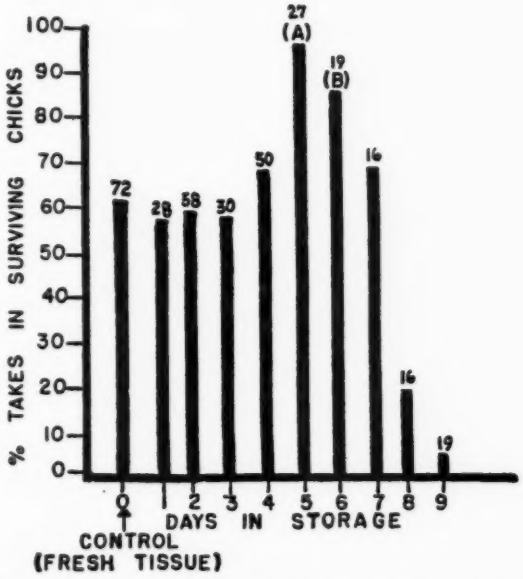
Tumor removed from the rabbit was stored in vials or petri dishes under the conditions indicated in table 1 and then transplanted to the CAM. The percentages of growths ("takes") were approximately equal for tumor stored for 7 days at 4° C., 11 days at −79° C., and for unstored tumor freshly explanted from the rabbit. However, the transplants stored at −79° C. rarely attained the minimal volume of 10 mm.^{3} during the permissible culture period (7–9 days). On the other hand, tumor maintained by Method 1 (storage under air, on a saline-moistened pad of gauze, at 4° C.) reached an average volume of 30 mm.^{3}, which is more tissue than is usually required to prepare 4 subcultures.

TABLE 1.—*Effects of storage under various conditions upon the subsequent growth of Brown-Pearce carcinoma on the chick chorio-allantois*

Method No.	Number of eggs*	Fluid added	Storage temp. °C.	Gas phase	Storage (*days*)	Percent takes†
1	263	saline‡	4	air	7	68§
2	32	none	−79, −10‖	"	3	7
3	12	"	−79	"	11	67¶
4	34	"	−10	"	2	0
5	15	"	−10	CO_2	1	0
6	13	"	−10	N_2	1	0
7	8	"	−10	O_2	1	25
7	8	"	−10	O_2	8	0
8	20	saline‡	17	air	1	60
8	15	"	17	"	3	7
8	12	"	17	"	4	17
9	17	"	38	"	1	19
9	16	"	38	"	3	0
10	72	"	controls**	"	0	63§

* Number of implanted embryos surviving 8 to 9 days after implantation.
† Percentage of living embryos with successful transplants.
‡ Tumor stored on gauze pad moistened with saline.
§ Average dimensions of growths: about 30 mm.^{3}.
‖ Tissue frozen at −79° C. with dry ice and stored at −10° C.
¶ Dimensions of growths usually below the minimum (3 × 3 × 3 mm.) required for multiple subcultures.
** Unstored tissue freshly explanted from rabbits.

In order to ascertain the optimal and maximal storage periods possible at 4° C. the tumor was transplanted to the CAM after storage periods ranging from 1 to 9 days (text-fig. 1). Tumor stored for 4 days at 4° C. showed no significant difference from fresh tumor in the percentage of takes. Storage for 5 or 6 days showed an apparent increase in the percentage of takes although there was no increase in the average size of the transplants. The tumor deteriorated after 8 days of storage as indicated by the low percentage of takes (approximately 20%).



TEXT-FIGURE 1.—Percentage of growths ("takes") of the Brown-Pearce tumor after various periods of storage at 4° C. The number of useful eggs (containing normal embryos) is indicated above each bar. Tumor stored for 5 days (*A*) shows a standard error of difference referred to 0 days of storage = 10 percent, t = 3.5, $P = 0.046$ percent. Tumor stored for 6 days (*B*) shows a standard error of difference referred to 0 days of storage = 12 percent, t = 1.9, $P = 5.7$ percent.

To ascertain the feasibility of prolonged culture on the CAM the tumor was cultured through 26 successive subcultures covering a period of over 200 days. For each subculture 8 to 12 eggs served as hosts. Eight or nine days after implantation of the tumor the percentage of dead embryos and the percentage of specimens showing successful growths (tumors exceeding 10 mm.^{3}) were recorded. The mortality of the chick hosts did not change during the culture period as shown by the percentages of dead chicks in the first and last 10 subcultures. The percentages of successful growths seemed to increase in the later subcultures. However, fresh tumor transplanted directly from the rabbit to the CAM shows such a high degree of variability in the percentage of takes (*5*) that conclusions

concerning enhancement of the tumor's capacity to establish itself on the CAM are not warranted.

Tumor carried through 19, 25, and 26 CAM subcultures was tested for its capacity to grow and metastasize in the mammalian host by reimplantation into the anterior chamber of the eye and the mesenteries. Tumor from the 19th egg subculture was implanted into the anterior chambers of both eyes of three rabbits. Intra-ocular growth occurred in all animals and visceral metastases in one. Tumor from the 25th and 26th egg subcultures was inoculated into the mesenterial and omental fat of one rabbit each. In both animals the tumor grew and metastasized within the 2-week period usually allotted for growth of the tumor transplanted from rabbit to rabbit. It is impossible to state whether growth and metastasis of the tumor had been altered quantitatively because of the high degree of variability in the growth and metastatic behavior of this tumor (*7*).

Discussion

The present results indicate that the Brown-Pearce tumor of the rabbit can be grown for at least 200 days, and perhaps indefinitely, on the CAM of the chick. However, if it becomes desirable or necessary to store the tumor for subsequent transplantation, storage conditions suitable for cultivation of the tumor in the rabbit are not necessarily suitable for the chick. Tumor stored at −79° C. may be grown in the rabbit provided that 5 or more weeks is allowed, but such tumor maintained on the CAM does not attain sufficient size for further egg cultivation. After storage at −10° C. for 1 or 2 days the tumor shows a low percentage of takes on the CAM, although it may be grown in the rabbit after at least 48 days of such storage.

Tumor stored for periods up to 7 days at 4° C. is approximately equal to fresh tumor both in its percentage of takes and in size increase during the permissible period of culture on the CAM. The apparently higher percentage of takes obtained with tumor stored for 5 or 6 days cannot as yet be regarded as true enhancement because of the high degree of variability found in both fresh and cold-stored tumor. Observations suggestive of enhancement after cold storage have also been reported by Walsh, Greiff, and Blumenthal (*8*) and by Mann (*9*). Sarcoma 37 stored at −30° C. for 14, 27, and 41 to 60 days showed shorter average latent periods and higher percentages of takes than fresh tumor or tumor stored at −30° C. for 1 to 7 days (*8*). Mouse carcinoma stored for less than 12 hours at −79° C. gave 41 percent takes, whereas tumor stored for 1 to 7 and 2 to 182 days gave 72 and 75 percent takes, respectively (*9*). However, these observations as well as the present results must be amplified before conclusions on the possible enhancing effects of cold storage are warranted.

Summary

The chorio-allantoic membrane (CAM) of the chick is a suitable environment for the continued cultivation of the Brown-Pearce tumor of

the rabbit. Tumor cultured continually on the CAM for over 200 days showed no apparent pathologic effects upon the chick host and was capable of approximately normal growth and metastasis in the rabbit.

The Brown-Pearce tumor may be stored for periods up to 7 days at 4° C. without appreciable decrease in the percentage of successful growths on the CAM. Tumor stored at −79° C., −10° C., 17° C. and 38° C. was unsuitable for growth on the CAM.

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Tumors of the Human Nervous System in Tissue Culture. I. The Cultivation and Cytology of Meningioma Cells [1, 2]

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The technique of tissue culture is useful because it provides us with the opportunity of studying the living components of the organism under special conditions of structural simplification. This simplification reaches such a degree that, following Albert Fischer's suggestion (*1*), we should consider only four basic morphologic types among all cellular elements capable of growth in cultures: 1) flat cells, spread in a continuous monostratified sheet and grouped in mosaic fashion (epithelial-tissue pattern); 2) cells with few prolongations, anastomosed in a tridimensional network (connective-tissue pattern); 3) cells provided with long, filamentous and mostly free prolongations (nervous-tissue pattern); and 4) isolated cells moving freely in the tissue-culture medium by means of active undulating membranes (non-tissue-forming pattern).

Since simplification of tissue structure is constant and permanent during cultures, and because it repeats itself with identical characteristics during many pathologic processes and constitutes the anatomical substratum in the involution of the organism, it becomes a phenomenon of special biological significance. When the tissue is stabilized *in vitro* and its cells are adapted to the experimental situation they show only those morphologic features which are intrinsic, thus permitting study of their intimate nature. In no other way can we better show histologic characteristics since in tissue cultures the cells are relieved of some of the influences exerted by neighboring tissues or cells and, therefore, they achieve a peculiar autonomy. For this reason cells outside the organism soon adopt the morphology of the corresponding archetype.

It is not easy to understand the biologic meaning of these archetypes, but it can be done to a certain extent by keeping in mind that structural

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simplification of cultured tissues is a precise and invariable phenomenon by means of which all details accessory to morphologic differentiation are lost and the cells show only their generic functional activity. Such a phenomenon represents a particular form of tissue metaplasia, a *nomicoplasia* (*νομικος* = legal, according to law) which is not progressive as prosoplasia, nor regressive as anaplasia. It is the permanent expression of the still little known laws ruling the natural evolution of cells during the development of the organisms.

Nomicoplasia tends to transform all connective elements into fibroblasts, thus creating complex problems in the interpretation of results obtained by means of explantation. It is certain that many fibroblasts which migrate *in vitro* originate from fibroblasts present in the explant by easy and continuous multiplication. However, the works of Levi (*2, 3*), Corner (*4*), Maximow (*5*), Fischer and Parker (*6*), Olivo (*7*), Bofill-Deulofeu (*8*), and others prove that all connective-tissue cells are capable of producing fibroblasts in tissue culture. This explains why Parker (*9,10*) was able to demonstrate quantitative differences of fibroblasts in culture, both in their fine morphology as well as in some of their functional reactions according to their source of origin.

This paper is concerned with our study of the meaning of nomicoplastic transformations shown by meningioma cells cultured outside the organism. Since nomicoplasia is a phenomenon common to all tissues, results of general application may be obtained by analyzing the simplification of the meningiomas *in vitro*.

Materials and Methods

The surgical material obtained by Dr. S. R. Snodgrass or one of us (I. J. J.) was sent from the operating room to the Tissue Culture Laboratory in sterile petri dishes. The tissue was then divided. One facing surface was used for culture and the opposite portion was sent to one of us (I. C.) for metallic stains and study.

Cultures were prepared according to the methods described by Parker (*11*) and Cameron (*12*). Fragments of tissue no more than 2-mm. square in surface and less than ½-mm. thick were embedded in an avian plasma clot. This material was then placed in roller tubes containing a nutritive fluid composed of 50 percent ascitic fluid, 45 percent Gey's balanced salt solution and 5 percent chick-embryo extract. The minimum time for incubation before study with phase-contrast optics, staining with aniline dyes, or silver impregnation was 24 hours, and the maximum time was 93 days (table 2). The explants were made in the course of other studies on human nervous tissue for which the following specimens were used. There were 70 specimens of cortex from prefrontal lobotomies, 97 intracranial and intraspinal tumors of varied histologic nature, and 16 specimens of cortex of various cerebral diseases. In addition, cultures were made of the normal meninges of a monkey and of a patient without a tumor. These cultures allowed us to establish a fruitful comparison of results. A total of 893 meningioma fragments were explanted. More

than half were stained by Jacobson's method, a fourth with Bodian's silver impregnation, and the remainder with Río Hortega's method for connective-tissue fibers. Some cultures were photographed with time-lapse cinematography at from 1 to 8 frames per minute in order to study cellular movements.

Results

The pertinent clinico-pathologic data are summarized in table 1 for 19 meningiomas and 1 meningosarcoma. The patients' ages ranged from 23 to 72 years. The highest incidence was in the sixth decade. There was a clear predominance in females. Thirteen tumors were supratentorial, 1 parasellar, 3 from the cerebellopontine angle and 3 intraspinal. The longest clinical evolution was 15 years and the shortest 9 months. In 2 patients the tumor recurred once and in another there were 3 recurrences in a period of 12 years.

The histologic picture of the tumors was that accepted as characteristic for meningiomas in 18 cases, so there was never any doubt as to their nature. There were 8 nodular meningiomas. Some of the tumors were hard. The parenchymatous nodules were relatively large and profusely anastomosed to each other (fig. 1). The cut surface was grossly compact and homogeneously lobulated or granular. Other tumors were soft and friable. The nodules were small and scattered in the loose connective tissue of the stroma (fig. 2). There were many small granulations, especially when acervuli were abundant. In our material there was one such case (but without calcification), and two others with many acervuli of the psammoma type.

In the remaining 10 typical meningiomas the cellular distribution was clearly diffuse with parenchymatous cells homogeneously distributed throughout the scanty stroma. There were few or no whorls and they contained a variable amount of stratified calcifications. In 4 of these 10 diffuse meningiomas there were fibrous plaques of variable size distributed in a disorderly way throughout some areas of the tumor and they showed degenerative signs of neoplastic cells. Two other diffuse meningiomas were fasciculated due to a large number of fine collagenous bundles placed among the parenchymatous cells (fig. 3). Two tumors were considered as angiomatous meningiomas since they had many blood vessels with abnormal walls and a mucinous appearance of the stroma (fig. 4). The last two meningiomas, which completed our group, were made up of imbricated syncytial sheets (fig. 5).

Besides the 17 typical meningiomas described above there was also one encapsulated, intradural, extramedullary tumor extending from thoracic 9 to thoracic 11. This tumor showed a fasciculated structure with many fine, weakly acidophilic fibrils filling the spaces between the cells and it also contained some chromoblasts. In this case the diagnosis of meningioma was not definitely established. In one instance (case 9), malignancy was evident pathologically, and it was therefore diagnosed as meningosarcoma (fig. 6).

TABLE 1.—*Clinico-pathologic data of meningiomas cultured* in vitro

Case No.	Identification data	Name and age	Sex and race	Location of tumor	Size (*in mm.*)	Pre-op. history	Summary of microscopic structure
1	UH:5531-G S–26101	A.D. 53	♂ White	Left motor area	40 × 55	6 yrs.	Hard, nodular, many whorls, scanty stroma, some acervuli.
2	UH:8291-H S-30943	A.A. 39	♀ Mex.	Right cerebellopontine angle	30 × 40	9 mos.	Soft, nodular, whorls, scanty stroma, many capillaries.
3	UH:3735-G S-25323	A.S. 53	♀ White	L. olfactory groove	70 × 50	15 yrs.	Hard, nodular, psammomatous, with scanty stroma and few blood vessels.
4	UH:1712-H S-32157	R.B. 40	♀ White	R. tentorium cerebelli	40 × 30	3 yrs.	Hard, nodular, many whorls, abundant stroma and blood vessels, acervuli.
5	UH:11066-F S-26034	S.M. 27	♀ White	R. cerebellopontine angle	30 × 50	1 yr., 1 recurr.	Hard, nodular, many whorls and scanty, vascular stroma.
6	UH:1389-H S-30730	T.J.B. 52	♀ White	Intradural extramedullar at T-9 to T-11	36 × 14	3 yrs.	Fasciculated, abundant intercellular collagen, some chromoblasts.
7	UH:15619-H S-32145	M.M. 53	♀ White	L. anterior fossa	50 × 30	2 yrs.	Diffuse, few whorls, dilated capillaries, microcysts and acervuli.
8	UH:1641-H S-31757	M.G. 47	♀ White	R. olfactory groove	40 × 70	8 yrs.	Diffuse, highly vascularized, abundant acervuli and fibrous areas.
9	UH:3466-C S-30541	F.E.S. 49	♀ White	R. occipitoparietal	60 × 40	5 yrs., 1 recurr.	Meningosarcoma with microcysts, dilated blood vessels, scanty stroma, many mitoses.
10	UH:19405-H S-32801	C.O.E. 55	♂ White	L. temporoparietal	90 × 60	2 yrs.	Fasciculated, few whorls, many acervuli, hyalinized blood vessels.
11	UH:109-J S-38342	M.C. 23	♀ Negro	L. parasellar	30 × 25	1½ yrs.	Hard, nodular, few whorls, scanty stroma, acervuli.
12	UH:7239-I S-34862	S.G. 56	♀ White	R. parasagittal frontal	60 × 58	11 yrs.	Nodular, psammomatous, many whorls, partially fibrous stroma.
13	UH:8043-I S-35547	L.M.C. 43	♀ Negro	R. tentorium cerebelli	40 × 60	2½ yrs.	Angiomatous, stroma either mucoid or fibrous in different areas.
14	UH:9145-I S-36349	S.S. 66	♀ White	L. frontoparietal	50 × 40	4 yrs.	Angiomatous, hyaline stroma, few whorls, many microcysts.
15	UH:12348-I S-36927	J.N. 48	♂ Negro	R. middle fossa	60 × 50	7 yrs.	Hard, nodular, many normal blood vessels.
16	UH:12656-I S-37076	E.F. 36	♀ Mex.	L. parieto-occipital	60 × 50	1½ yrs.	Laminated, diffuse, hyalinized blood vessels, scanty stroma.
17	UH:12901-I S-37719	N.H. 56	♀ White	L. frontal	60 × 75	12 yrs., 3 recurr.	Diffuse, fasciculated, large fibrillary areas, some mitoses.
18	UH:10706-G S-27487	P.C. 72	♀ White	Extra and intradural T-3	30 × 15	2 yrs.	Dense, diffuse; next to it were large fibrous masses, apparently dura.
19	UH:7970-J S-41008	L.T. 54	♀ White	Extramedullar intradural T-9	10 × 30	1 yr.	Laminated, hard, many calcified concretions.
20	UH:9837-F S-27990	L.S. 62	♀ Negro	R. frontoparietal	50 × 30	3½ yrs.	Diffuse, polymorphic cells, hyalinized stroma and blood vessels.

Table 2 is an attempt to evaluate objectively the most important data obtained from the study of fixed and stained cultures. The relative intensity of changes has been arbitrarily divided into three degrees. These data are given for the different types of migrating cells and their grouping and functional activity are also shown. The minimum and maximum time of culture, as well as the general intensity of growth, for each case is also given.

In our cultures of meningiomas we have been able to identify elements corresponding to the connective tissue and non-tissue-forming patterns (table 2), but no epithelial- or nervous-tissue patterns were found. Within the aggregation of connective tissue we have been able to distinguish with sufficient certainty some elements belonging to the neoplastic parenchyma, such as endothelial (meningothelial) cells, fibroblasts and fibrocytes. Other connective-tissue elements were chromoblasts, giant cells, plasma cells and mast cells, and they have been considered extrinsic to the tumor but nevertheless appeared occasionally in some cultures. Within the non-tissue-forming archetype we have distinguished histiocytes, macrophases and leukocytes. Some inclusions of neurogenic elements will be described separately.

The analysis of results summarized in tables 1 and 2 has resulted in the following objective observations.

Intensity of Growth

The intensity of growth was judged by taking into account the rate and extent of cellular migration, the number of mitoses, and the presence and size of autolysis as represented by a zone of necrobiosis.

The vast majority of our cultures showed rapid and complete *cellular migration*. In more than half of the explanted fragments migration reached a maximum degree, forming on each cover slide a sheet of migrated cells which became continuous and homogeneous between 12 and 20 days after being placed in culture. One fourth of the fragments developed satisfactorily, although not in such a way as the preceding ones since the areas of outgrowth did not become fused into continuous sheets. In the remaining preparations there was poor growth or none at all, mainly due to technical accidents such as contamination.

Without any relation to the preceding description, in 7 cases there were a few or no *mitoses* in migrated cells. The frequency of cellular division showed two general peaks. One occurred between the 3rd and the 6th days and the other between the 35th and the 62nd days. Older cultures were usually richer in mitoses. In 4 tumors there was a moderate number of mitoses and in 9 others mitoses were abundant. In case 4, tripolar mitotic figures were observed which were not identified in corresponding histologic sections. Elements of mitotic activity were found in the following order: fibroblasts, histiocytes, endothelial cells and macrophages.

Three cases were characterized by rapid and intense *autolysis* in many parts of the area of outgrowth which did not interfere with the exuberant development of the remaining migrated elements. In 3 other menin-

TABLE 2.—*Results of tissue culture of meningiomas*

Case number	Cultures	Days in culture		Intensity of growth		Connective-tissue archetype							Non-tissue-forming archetype		
		Minimum	Maximum	Migration	Mitoses	Autolysis	Endothelium	Syncytium	Whorls	Aerovuli	Fibroblasts	Giant cells	Plasma cells	Mast cells	Histiocytes	Macrophages	Leukocytes
1	6	11	14	+++	+++	++	...	++++	...	+	+++	...	...	...	...	...	...
2	39	12	43	+++	+++	+	+++	+++	+++	+	+++	...	...	...	...	...	...
3	44	8	35	+++	+++	...	++	++	++	+	+++	...	...	...	...	...	...
4	80	7	90	+++	+	...	...	...	...	...	+++	...	...	...	...	...	...
5	8	14	23	+++	...	+	...	...	...	...	+++	+	...	...	+	++	...
6	52	8	70	+++	+++	...	...	...	...	...	+++	...	...	...	+	+	...
7	42	15	70	+++	+++	+	+	...	++	...	+++	+	...	...	+	...	...
8	78	12	40	+++	+++	+	...	...	...	+	+++	...	...	...	+	...	...
9	42	3	62	+++	+	+	+	...	...	...	+++	+	...	...	+	+	...
10	38	5	67	+++	+	...	++	...	+++	++	+++	...	...	...	+	+	...
11	13	4	41	+++	+	...	++	...	++	+	+++	...	...	...	+	+	...
12	21	19	93	+++	+	...	++	...	++	+	+++	...	...	...	+	+	...
13	20	18	38	+++	+	...	+	...	...	+	+++	...	+	...	+	+	...
14	30	4	42	+++	+	+	+	...	++	+	+++	+	...	...	+	+	...
15	85	10	28	+++	+	+	...	+++	+	+	+++	...	...	+	++	++	+
16	54	4	15	+++	+	...	+	...	...	...	+++	...	...	...	...	+	+
17	149	4	48	+++	+++	+	+	+	++	+	+++	...	...	...	+	+	+
18	10	7	88	+++	+++	...	+	...	...	...	+++	...	...	...	...	...	...
19	74	1	42	+++	+++	+	+	+	+	+	+++	...	...	...	+	+	+
20	8	26	31	+++	+	+	...	...	...	...	+++	...	...	...	...	...	...

giomas there were minor areas of autolysis, and, in 6 others, autolysis appeared as a late and minimal phenomenon.

Endothelial Cells

In less than half of the explanted tumors endothelial cells appeared in what might be called pure cultures, but they remained only during the first days of incubation. Ordinarily, by the end of the initial 24 hours the first fibroblasts appeared through nomicoplasia in nearly all explanted fragments. They were so numerous by the 12th to the 16th days that they dominated the picture to such an extent that endothelial elements were hardly recognizable. For this reason, it is believed that there were endothelial cells in many of the other cultures, but they were not seen after fixation and staining since this was done at later stages.

Endothelial cells that proliferate in cultures of meningioma are small when compared to the fibroblasts. They are narrow and elongated, and are very similar in form and size with one another. They tend to arrange themselves in wide, loose fascicles or in narrow, dense cords (fig. 7). Between fascicles and cords there are wide spaces occupied by similar elements arranged in a tridimensional network through delicate intercellular anastomosis. One observes in many of these reticular areas of outgrowth the tendency of epithelial cells to become oriented with their major axes following a predominant direction. Sometimes endothelial sheets cross each other without losing their particular orientation. Endothelial cells proliferated in cultures of meningioma occasionally preserve the specific arrangement of the tumor cells, *i.e.*, they show syncytia, whorls and stratified calcifications and, therefore, are easily recognized and identified *in vitro* as meningothelial cells.

The meningothelial *syncytia* also appear early in the explants, but they are present for a longer time than free endothelial cells. These syncytia appear in tissue cultures with a relatively high frequency (8 of our cases) and show a curious polymorphism. Some are small, either stellate or arranged in parallel tracts, and contain from 3 to 12 nuclei. Others are quite large with several dozen to a hundred nuclei and tend to become reticulated in the center (fig. 8). When these large syncytia are carefully examined it is easily seen that cells anastomosed in networks are detached from them, either by a sliding of the superficial protoplasmic sheets or by the loosening of the central or deeper areas. All degrees of transition are observed between large and small syncytia. The syncytial state is not permanent in tissue culture and, as described by Río Hortega (*13*, *14*) in histologic sections, the syncytia when present within the explants become isolated cells which, in turn, change readily into fibroblasts.

Characteristic *whorls* were found in 12 of the cultured tumors. They were similar to those present in histologic sections of typical meningiomas and have been compared, not without reason, to the concentric cellular groupings of Pacchionian bodies in the normal leptomeninges. It was convenient to distinguish two principal types of whorls observed *in vitro* (figs. 9 and 10). Some were large and made up of many small cells with

highly chromophilic nuclei; others were small, formed by two to six cells with a large amount of cytoplasm arranged around a basophilic, hyaline mass which was a degenerated central cell. Still others showed all these morphologic features.

Acervuli were unchanging structures which when present in the explants followed the migrating cells in their wanderings. The surrounding elements for some time conserved their concentric arrangement around the calcifications but sooner or later they lost contact with the acervuli and then the arrangement was entirely lost. In these calcifications we never observed any organic structures which might be taken as the specific center of formation. On the contrary, we believe that they start in amorphous cellular debris.

Fibroblasts

Fibroblasts developed in all cultures showing any growth. This development was maximal in 16 cases, moderate in 2 and poor in the remaining 2 (table 2). Accompanying these fibroblasts in the cultures, one can always find intercellular fibers which are stainable from the early stages of their growth with the method of Río Hortega. In older cultures these fibers took up the eosin in Jacobson's staining method with sufficient intensity to enable their easy visualization and at this time exhibit the histochemical properties of collagen (figs. 11 and 12). Maximal development occurred, however, some time later between the 12th and the 42nd days of culture. On the other hand, in 10 of the explanted tumors we found varying numbers of connective-tissue cells containing intracytoplasmic fibrils of variable shapes and inconstant staining properties. These fibrils were most outstanding in preparations stained with Bodian's method. They were equally visible—in large numbers in some cases—with the use of Jacobson's method, although they took up the stain weakly. They could also be stained by the silver carbonate method of Río Hortega and almost always acquired shades of color which differed from those seen in the intercellular fibers.

In the specific case of meningiomas it is relatively easy to differentiate fibroblasts from their more immediate neighbors, the endothelial (meningothelial) cells. As has been stated, endothelial cells are smaller, longer and, above all, often preserve in culture their characteristic tendency to arrange in fascicles, to become fused in syncytia, to group in whorls, and to contain acervuli. Comparing figures 7 and 8 with figures 11 and 12 [the former corresponding to endothelial (meningothelial) cells and the latter to fibroblasts, both stained with the same method and photographed at the same magnification], we see a clear difference in shape, size and arrangement. Furthermore, between fibroblasts there were collagenous fibers which were not seen among the endothelial cells. However, as can be observed in the same figures, there were frequent transitions between endothelial and fibroblastic cells. This is due to nomicoplastic simplification in which the latter arose from the former in the vast majority of our meningioma cultures. Definite identification of fibroblasts

in vitro, therefore, can be made only by showing intercellular reticulum with appropriate silver-impregnation techniques which is a product of their own functional activity.

Other Connective-Tissue Elements

Besides endothelium and fibroblasts the study of stained meningioma cultures has enabled us to recognize other cells of the connective-tissue pattern such as chromoblasts, giant cells, plasma cells and mast cells.

Chromoblasts were only seen in case 6, in which they were also noticeable in sections. Their presence has a negative value with reference to meningioma, since the growth of this tumor did not correspond to the growth of the others in this series but to the growth of neurinomas. This diagnosis was later confirmed.

Giant cells were present in three of our tumors in culture. In case 6 they were found in fixed preparations after 5 weeks' outgrowth *in vitro*. They had between 20 and 80 nuclei, vacuolated chromophilic endoplasma, and very delicate, poorly outlined exoplasm. In all probability they were polynucleated, neoplastic elements similar to the ones frequently seen in sections of neurinomas. In case 9, giant cells were seen only before the third week and grew from the explant as thick protoplasmic buddings containing approximately 20 central nuclei. Later they became rounded cells with unequally vacuolated cytoplasm and with nuclei arranged in a dense and well-defined ring. These cells produced a dense and acidophilic mass within the space limited by the nuclear ring and later a very delicate, foamy exoplasm extended to some distance. Giant cells appeared in case 14 on about the 21st day of culture presenting between 12 and 60 peripheral nuclei arranged in a crescent, as in Langhan's cells, and with a very dense and homogeneous cytoplasm that was sharply defined. In the last two cases we believe that the giant cells arose from tumor cells and represented an accidental and transitory form in their nomicoplastic transformation in the direction of fibroblast formation.

We found *plasma cells* only in 4-day cultures of case 14. They were located near blood vessels at the margin of the explant. Leukocytes and red blood cells were still visible within the lumen of the vessel, representing elements persisting with the explanted fragment. *Mast cells* were also found in only one case and were preserved up to the 12th day, but we never saw them outside of the explant.

Non-Tissue-Forming Archetype

In the early history of two cultures, *histiocytes* of characteristic shape and arrangement were the dominant cells in the outgrowth rather than endothelial and fibroblastic elements. They were moderately abundant in four other cases and were recognized as isolated individuals in one other case. They were not seen as such after the 12th day, finally becoming vacuolated macrophages or fibroblasts.

Macrophages are seen more easily in time-lapse, phase-contrast motion

pictures of living cultures than in stained preparations. The refringency of their characteristic vacuoles and inclusions and the active movements of their undulating membranes disappear after fixation and may be overlooked. This is probably the reason why only vacuolated macrophages were recorded in the preparations of five cases, and in four of them they were present in unimportant numbers (table 2). We believe that in most instances macrophages arise from pre-existing histiocytes, and that they tend to disappear, becoming fibroblasts when the solid phase of the culture medium maintains a propitious consistency. The nourishment of migrated cells must be satisfactory.

In three cases there were abundant *leukocytes* in the cultures. They always migrated rapidly, reaching a considerable distance from explant in a few hours and remaining there for many days before undergoing autolysis. In four other cases leukocytes were only occasionally discovered and in small groups. They always represented pre-existing elements.

Nervous-Tissue Archetype

The presence of neurogenic cells in our cultures of some meningiomas caused serious difficulties. In order to understand this it should be remembered that there are many who accept the views of Oberling (*15*, *16*) regarding the ectodermic origin of meningoblasts, and that Roussy, Cornil and Leroux (*17*) speak of "tumeurs méningées à type glial." Another source of confusion was the presence in 16 of our cases of ramified fibrocytes containing desmofibrils, often not too different from neurofibrils and neuroglia fibrils.

Our problems started with case 2 where, in some slides, there were isolated neurons and nerve fibers. This was again observed in case 5 where astrocytes were also discovered. Thanks to the transparency of the explants, we found conclusive evidence that such neurogenic structures originated in the peritumoral nervous tissue set up in culture with the neoplastic cells. Therefore, we were even more careful to be certain to explant deep portions of pure tumor and, separately, a portion of adjoining nerve tissue when the latter was part of the surgical specimen. This was done in cases 3, 4, 5, 8, 10 and 15.

Furthermore, the simultaneous culture of normal and pathologic nervous tissue obtained from other patients was useful in increasing our experience. With this experience we were convinced that in our meningioma cultures there were never any cells of nervous-tissue archetype arising from neoplastic elements, and were also able to identify with relative facility the Schwann cells that developed in case 6, which provisionally was diagnosed as a meningioma.

Meningosarcoma

Case 9, illustrated in figure 6, was a meningosarcoma comprised of small cells with delicate cytoplasm, abundant desmofibrils, frequent mitotic figures, a very fine reticular stroma, and many blood vessels without a

wall of their own. The diagnosis made in surgical pathology after the first operation, which was performed a year before the second one, was of meningioma. In cultures, this malignant tumor did not behave differently from the remaining meningiomas. However, growth was much faster and the transformation of neoplastic cells into fibroblasts and fibrocytes was so rapid that there were intercellular acidophilic fibers on the 3rd day of culture. Syncytia, whorls or acervuli were not seen but there were abundant neoplastic elements similar to histiocytes in that they developed exoplasmic veils resembling undulating membranes, though these were larger and less active. Desmofibrils were well preserved *in vitro* and it is certain that new ones developed. During the first days of culture, migrated neoplastic cells showed a highly granular chromatic network and a clear perinucleolar halo which were absent in cultures of the other meningiomas. This was one of the three cases in our series where there were giant multinucleated cells. Tumor cells elaborated proteolytic ferments that produced liquefaction in many portions of the solid phase of the cultures.

Non-Neoplastic Meninges

Tissue from a non-neoplastic specimen not included in this series and from the meninges of a normal monkey cultured for comparative purposes allowed us to demonstrate that normal collagenous bundles of the dura do not change their histologic features when maintained *in vitro* for periods up to 47 days. Normal meningeal cells produce reticular fibers with greater speed, energy, constancy and uniformity than meningioma cells, perhaps because in the normal meningeal cultures there were abundant fibroblasts capable of initiating fibrogenesis without undergoing nomicoplasia from endothelium (meningothelium).

Discussion

The first papers published on the cultures of meningiomas by Kredel (*18,19*), Buckley and Eisenhardt (*20*), Cox and Cranage (*21*), and Wolf and Honeyman (*22*) showed meager results due to the inadequacy of the methods employed. The low consistency of the culture medium and the frequent resetting in many cases hindered development and caused a prevalence of migrating elements in those few that showed any outgrowth. These papers deserve the merit of having initiated attempts to maintain intracranial tumors *in vitro* but their conclusions are not very valuable.

Bland and Russell (*23*) set up technically adequate cultures which yielded positive results. The microscopic picture of meningiomas, so varied in histologic sections, was translated into a single pattern which was described as beginning with "epithelioid but no true epithelial sheets." This undoubtedly corresponded to meningothelium. Outgrowth was described as ending with cells containing fibrils and arranged in various forms which corresponded to fibroblasts. Unfortunately, Bland and Russell did not find whorls or syncytia, perhaps because they explanted fragments that were too large and because they subjected their cultures to unnecessarily frequent changes of the nourishing medium.

The paper of Murray (*24*), although based on only four tumors, contains valuable conclusions. She recognized the endothelial nature of migrated neoplastic elements, stained the argyrophilic cement borders among them, and observed and described intracytoplasmic fibrils and "desmoblasts." She claimed that meningioma cells do not form reticular fibers in culture, probably because she considered, quite logically, that the endothelial cells were the only neoplastic elements.

Adaptation of Meningiomas In Vitro

Using an adequate culture technique, meningiomas are highly adaptable to short-term experiences *in vitro*. As can be seen in table 2, migrated cells grow easily and show a minimum of regressive phenomena attributable to the method of study.

There are no doubts regarding the neoplastic nature of the endothelial cells developed during the first few days in the culture of meningiomas. On one side, the transparency of the explant will frequently allow direct observation of migration of elements of tumor parenchyma, and this can be seen in the upper left corner of figure 8, representing a small area of the margin of the explant. Cells situated in the explant penetrate the nutrient medium, thus preserving their morphologic and tinctorial characteristics, and show no more variations than those usually seen in all kinds of cultures; *i.e.*, moderate flattening on the surface of the plasma clot. On the other side, it is universally accepted that syncytia and whorls, as formed by migrated cells, are characteristic structures of meningothelial cells.

The rhythm of mitotic divisions in meningioma cells observed by us, as well as the presence of tripolar figures in one case, do not seem to depend directly on the neoplastic nature of the explanted material. The transformation of meningothelial cells into fibroblasts and the initiation of fibroblastic activity perhaps initially slow the rhythm of cellular division, which is high during the early hours of migration. It is possible, too, that the eventual inactivity of fibroblasts in old cultures, as well as the presence of products of autolysis, restimulate the multiplication of migrated cells. This is our explanation of the two peaks of maximal intensity of mitotic figures shown by the curve toward the 3rd and 6th days and between the 35th and 62nd days of culture.

Autolysis is a phenomenon common to many types of cells in culture. It is greater in malignant tumors as was the case in our meningosarcoma. Frequently, cellular autolysis is accompanied by great liquefaction of the solid phase that consists of coagulated chick plasma and embryonic extract, which is very sensitive to proteolytic enzymes.

Histologic Nature of Meningiomas

The presence of cellular archetypes in tissues cultured outside the organism parallels modern tissue classifications as studied with classic methods. According to histologists there are four fundamental tissues in higher animals: epithelial, connective, muscular, and nervous. These

divisions, so useful for the classification of tumors, can be made even simpler by reducing them—as suggested by the results of the tissue cultures—to only three basic histologic types: epithelial tissues, formed exclusively by cells; connective tissues, having the ability to form filamentous intercellular substances of collagenic nature; and nervous tissues, made up of cells with long filamentous processes which have free ends. Cells coming from each of these three basic tissue types may behave as migrating elements under normal or pathologic circumstances. Regardless of their variable origin, free migrating cells never constitute a genuine tissue. Being a part of the supporting system, muscular tissue may be considered a special variety of connective tissue.

That morphologic concept brings up immediately the problem of deciding the correct site among the three types of true tissues mentioned for endothelium. No doubt endothelia are pure cellular sheets and, therefore, are histologically epithelial. However, in pathologic situations, especially in endotheliomas, endothelium does not behave permanently as epithelium. Thus, at the beginning meningiomas contain large masses of neoplastic cells without fibrillar intercellular substance, but later, if their natural evolution proceeds, malignant forms also include very abundant reticular fibers, while neoformed collagenous bundles appear everywhere. The same can be said of many other connective tumors since their cells also undergo nomicoplasia and show an increasing number of fibroblasts with time.

All this means that endothelia exhibit an epithelial texture as long as they retain their role of lining surfaces. When the lining function is lost they assume a fibroblastic activity; these are characteristically connective property. Meningothelial, as well as meningioma cells, are different from fibroblasts since they never form intercellular connective fibers without previously undergoing nomicoplasia, that is, without loss of their endothelial nature. All connective tumors are capable of progressively forming reticular fibers through nomicoplasia of their specific elements. However, the term fibroblastoma was created to designate only those tumors formed by active or true fibroblasts; in other words, tumors containing fibers among all their cells deriving directly from neoplastic cells. This is not the case of meningiomas. In consequence, after establishing in this paper the properties in meningioma cells, emphasized by Mallory (*25*), we cannot consider meningiomas as fibroblastomas.

Embryonic Origin of Meningiomas

The tendency to form syncytia and whorls gives special characteristics to the meningothelium which is enough to separate it from other endothelia. The observation of some whorls in histologic sections of meningiomas and, above all, those formed *in vitro* (figs. 9 and 10) leads to the acceptance of Cleland's hypothesis (*26*). He was the first to consider Pacchionian bodies as the most important sites of origin for the development of meningiomas, a hypothesis with which the majority of present-day authors agree.

A very different matter is the one referring to the embryonic origin of the meningothelium and, therefore, of meningiomas. Since the time of Cruveilhier (*27*), and until fairly recently, it was accepted that meningiomas are connective or endothelial (epithelial) tumors or, in any case, of mesenchymal origin. After the work of Sterzy (*28*), no one doubted the mesodermal origin of the membranes surrounding the brain and spinal cord. However, while studying the development of *Amblystoma* and *Rana palustris*, Stone (*29, 30*) and Oberling (*15, 16*) in analyzing the structure of meningiomas submitted the hypothesis that meningoblasts arise from the neural crest and therefore are of ectodermal origin. In our opinion, this is a very delicate problem that belongs to the domain of embryologists.

Certainly the histopathologic study of meningeal tumors, the behavior of meningiomas and also of normal meninges in tissue culture are not adequate to provide arguments of such a nature as to confront the findings obtained by Weed (*31, 32*), Harvey and Burr, Flexner, Raven, Harvey, Burr and Capenhout, Essick (bibliography in Foot *33*) and Sensenig (*34*), who made observations directly on embryos of various animals without being able to solve the question. In any case, whether the meninges are exclusively mesenchymatous or whether their endothelium originates in the neural crest, meningiomas should be considered as endotheliomas, following the classic idea of Golgi (*35*), who regarded them as tumors formed by connective cells and maintained in an epithelial state because they were lining surfaces but which regressed to active fibroblasts under special circumstances in which they had the opportunity of showing their genetical potentialities.

Origin of Fibroblasts in Cultures of Meningioma

It is not easy to answer in a definitive way the question of the origin of fibroblasts developed in cultures of meningiomas. This problem is important because it repeats itself with identical characteristics in all kinds of cultures, with the possible exception of the lens. It is generally agreed that explanted fibroblasts, even when they are very few, will multiply *in vitro* with such facility that in the end they will dominate all the remaining cellular species in the same culture. Such multiplication of pre-existing fibroblasts doubtlessly occurs in cultures, but it will not suffice to explain by itself the absolute predominance of fibroblasts or other phenomena that are simultaneously observed.

Thus, when we study the cellular growth in all types of cultures we observe that the behavior of fibroblasts is different according to the histologic nature of the parenchyma of the explanted organ. When there are in the explant a nonconnective parenchyma and a fibroblast containing stroma, parenchymatous cells are well individualized in an almost permanent way in all slides examined in short-term cultures (100 days as a maximum). Furthermore, in some of these cultures fibroblasts may never develop, or they may appear in small number, as if they had lost the special capacity to multiply. On the other hand, when the explant is

derived from a mesenchymal organ, regardless of the cellular species that it may contain, the same phenomenon described in cultures of meningiomas is observed; *i.e.*, parenchymatous cells migrate during the first days of culture. However, such differentiated, parenchymatous cells entirely disappear and are replaced by fibroblasts. Some of these fibroblasts may originate from those which migrated from the stroma at the time that parenchymatous cells migrate, but in the majority of the slides they will not appear until later in the explant. In any case, the end result is always the same: after 5 weeks of culture—and sometimes much earlier—all we could see were fibroblasts. Unless we accept that parenchymatous cells of mesenchymal origin succumb inadvertently, we will have to admit that, at least in part, they gave origin to fibroblasts, which are the only inhabitants of the slides during the last phases of culture.

There is additional evidence to accept nomicoplasia as a fact, through which all connective elements not excessively differentiated may change into fibroblasts: first, the previously mentioned results of other authors (*2–4, 6–10*) and especially the presence of fibroblasts actively elaborating collagen bundles from blood clots that contained originally nothing but white blood cells (Maximow *5*) [In this particular case, it is not possible to count on pre-existing fibroblasts in order to explain the origin of those that will occupy all the preparation in a few weeks.]; second, the existence of obvious transitions between other explanted connective-tissue cells and the late fibroblasts in the same culture, as shown in figure 11 otherwise cannot be explained. Such transitions are never demonstrable when the parenchyma of the explanted organ is not mesenchymal, at least in short-term cultures. On the contrary, epithelial and neurogenic cells will develop in cultures permanent areas without fibroblasts, even in a case when in other parts of the slide there is abundance of fibroblasts.

Finally, we believe that the wrong use of the word is the main reason why the origin of fibroblasts in tissue culture is difficult to interpret. However, Río Hortega's method provides us with a sure, easy and constant technique for impregnating precollagenic, reticular fibers. When used as a routine it is possible to judge objectively the phenomena of fibrogenesis. Only then can we discriminate as fibroblasts the particular cells involved in elaboration of reticular fibers. Myoblasts from the heart of the chick embryo, as well as osteoblasts, chondroblasts, histiocytes, endothelia, mesothelia, meningothelia, etc., originating in other histologic varieties of connective tissue do not elaborate reticular fibers, even if they morphologically resemble fibroblasts in tissue cultures. When reticular fibers appear in cultures containing the former cells, we always find other cells with size, grouping and structure of real fibroblasts. Between these new fibroblasts and the remaining preceding connective-tissue cells one can see, at certain times, all the corresponding transitions, as we have described them for meningiomas in this paper. The transformation of mesenchymal cells into fibroblasts (nomicoplasia) is, therefore, a much more generalized, effective, and constant phenomenon than the persistent multiplication of pre-existing fibroblasts. The latter type of proliferation

only rarely if ever can produce, by itself, the absolute predominance of fibroblasts in cultures of organs with nonmesenchymal parenchyma.

Fibrogenesis, however, as a property peculiar to connective tissues, is too complex a process to be discussed here in detail. Therefore, the next paper of this series will be dedicated to analyzing the morphology of fibrogenesis in tissue cultures of the same cases of meningioma.

Summary

1) Meningioma cells migrating in culture medium behave as endothelia (meningothelia) and often preserve their capacity to fuse into syncytia and to form whorls. There are no essential differences in the morphology of cultures obtained from the various histologic varieties of meningioma. Only meningosarcomas grow faster and show some other secondary features related to the low maturity of neoplastic cells.

2) In cultures of meningioma, giant cells may appear representing transitory forms of neoplastic elements. Plasma cells, mast cells, histiocytes, macrophages and leukocytes are present as extrinsic and preexisting elements. Nerve cells, nerve fibers and astrocytes develop only when adjacent areas of nervous tissue are explanted together with the tumor.

3) Neoplastic cells in this series became fibroblasts between the 3rd and 42nd days of culture. This transformation is not limited to endothelial cells in meningiomas, but is common to all explanted connective-tissue cells. The natural transformation of connective-tissue cells into fibroblasts represents a phenomenon of morphologic and functional simplification—a legitimate metaplasia or nomicoplasia symbolic of the laws governing the natural evolution of cells in the organism.

4) Meningioma and meningosarcoma behave *in vitro* as connective tumors of endothelial texture. Histopathology and tissue culture fail to provide data that may decide upon the mesodermal or ectodermal origin of meninges and meningiomas. These tumors deserve such characterization because they are the only neoplasms forming syncytia and whorls. Some neoplastic whorls even reproduce the exact structure of Pacchionian bodies, which means that meningiomas are the specific tumors of the meninges. Tumors similar to those which develop in other organs, even when they occur in the meninges, should not be classified as meningiomas.

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PLATE 85

Main histologic varieties of cultivated meningiomas. Hematoxylin and eosin. × 70

FIGURE 1.—Hard, nodular meningioma. Case 1.

FIGURE 2.—Soft, nodular meningioma. Case 2.

FIGURE 3.—Diffuse fasciculate meningioma with fine collagenous bundles. Case 17.

FIGURE 4.—Angiomatous meningioma. Case 13.

FIGURE 5.—Laminated, syncytial meningioma. Case 16.

FIGURE 6.—Meningosarcoma; one year earlier the structure was of a meningioma. Case 9.

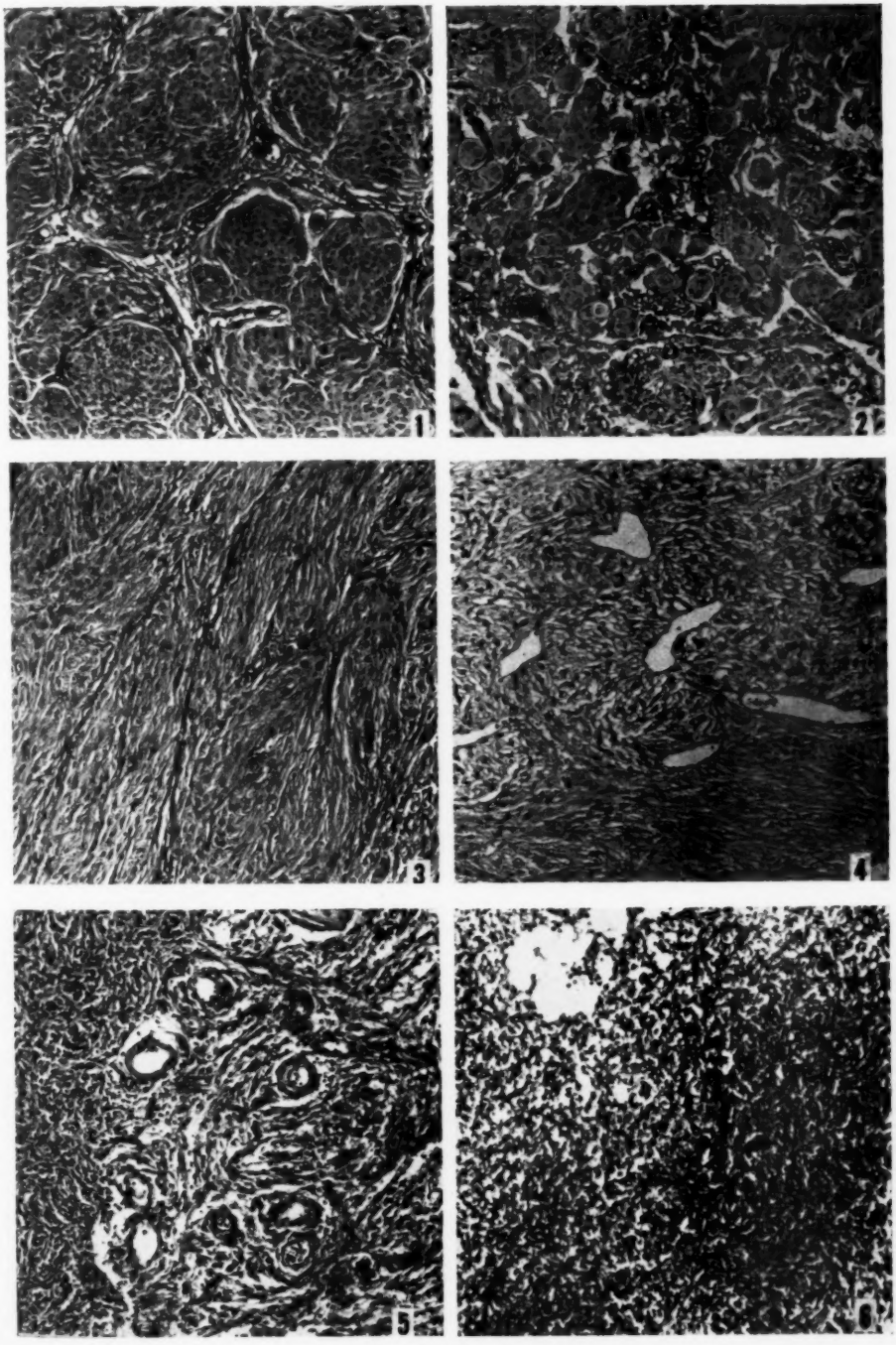

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PLATE 86

Endothelial (meningothelial) cells and fibroblasts which have migrated in cultures of meningioma.

FIGURE 7.—Typical endothelial morphology and arrangement of migrated meningioma cells. Case 19. (S.III–F.1.) 4-day culture. Jacobson's. × 110

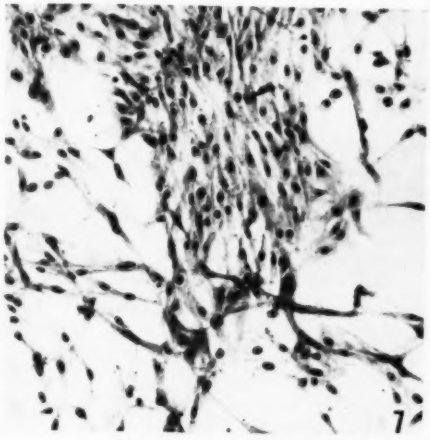
FIGURE 8.—Medium-sized syncytium formed by migrated meningioma cells. Case 19. (S. III–F.4.) 4-day culture. Jacobson's. × 110

FIGURE 9.—Large and small whorls formed by migrated meningioma cells *in vitro*. Case 3. (S.XX–F.3.) 21-day culture. Bodian's. × 110

FIGURE 10.—A detail of meningioma cells forming whorls in tissue culture. Note the similiarity to Pacchionian bodies. Case 3. (S.XX–F.3.) 21-day culture. Bodian's. × 220

FIGURE 11.—Noimeoplastic transformation of meningioma cells with an endothelial texture into fibroblasts; connective fibers are only visible around fibroblasts. Case 8. (S.XVIII–F.1.) 18-day culture. Jacobson's. × 110

FIGURE 12.—Another field similar to the previous one showing only fibroblasts and connective intercellular fibers. Case 8. (S.XVIII–F.4.) 18-day culture. Jacobson's. × 110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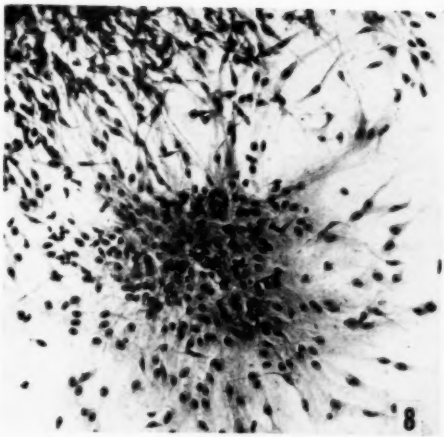
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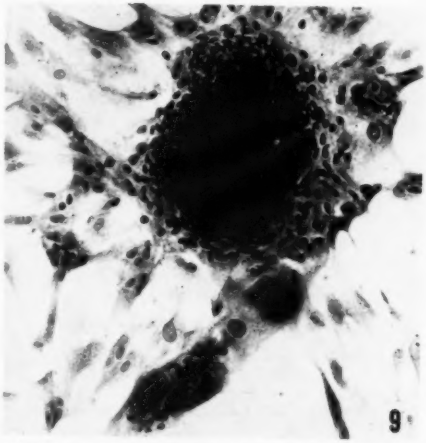
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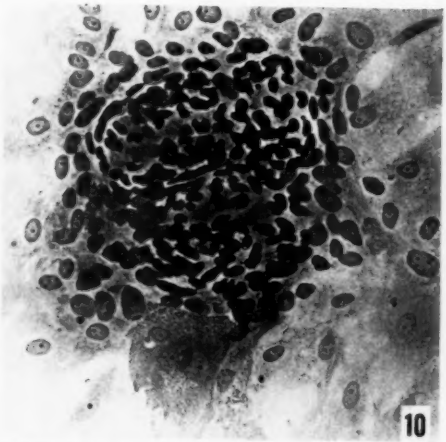
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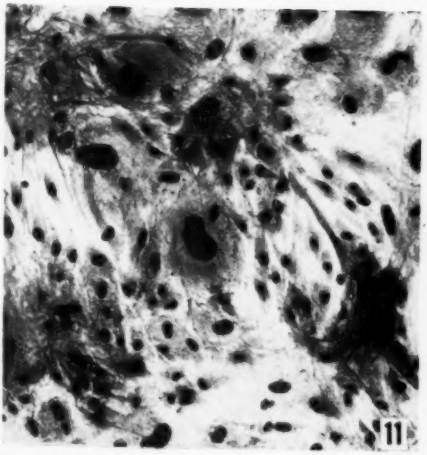
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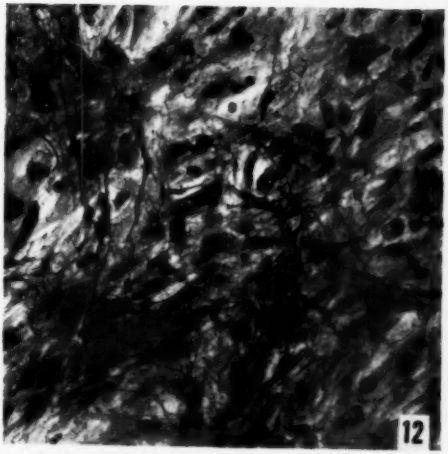
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Tumors of the Human Nervous System in Tissue Culture. II. An Analysis of Fibroblastic Activity in Meningiomas [1, 2]

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In the first paper of this series (*1*) we have studied the behavior of 20 meningiomas cultivated outside the organism in roller tubes. In those cultures we obtained abundant neoplastic cells that appeared *in vitro* as endothelial cells, with the pecularities of whorl and syncytia formation in the areas of growth, identical to those characteristic of meningiomas in histologic sections. Furthermore, in some cases we also identified giant cells, plasma cells, mast cells, histiocytes, macrophages and leukocytes, and the significance of this finding was also discussed.

The most outstanding feature was, however, the relatively rapid disappearance of migrated neoplastic cells, which were replaced by fibroblasts. In 16 of those 20 cases, fibroblasts developed with such intensity that they completely covered the slide in short-term cultures (100 days as a maximum). They formed a continuous sheet in which the outlines of the different explants were no longer visible. In two more cases the growth of fibroblasts was moderate and in the two remaining it was considered as slight.

The mentioned predominance of fibroblasts is not a phenomenon peculiar to cultures of meningioma, but is repeated whenever tissues of exclusively mesenchymal elements are explanted. As soon as five weeks after the explant, and sometimes even much earlier, all one can see in the slides is fibroblasts. This fact is apparently not explained only by the easy and uninterrupted reproduction of pre-existing fibroblasts in the explant. Whether there are fibroblasts or not in the explant, migrated connective cells, and especially endothelial (meningothelial) cells, will become fibroblasts through a process of legitimate metaplasia or nomicoplasia.

Meningothelial cells, as all endothelia do, undergo such nomicoplasia with extreme facility. Therefore, cultures of meningioma are very ade-

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quate material for the analysis of the morphology of active fibroblasts and for the direct study of all phases of fibrogenesis. Much insight can be gained into the still unanswered question of the origin of collagen fibers. At the same time, new fields of support can be provided for recent advances in the histochemistry of connective tissue [see Ragan (*2*)] and for the role played by the intercellular, filamentous substances in the so-called collagen diseases [Klemperer (*3*)]. Therefore, we have dedicated this paper to a study of fibrogenesis as it is observed in cultures of meningiomas.

Material and Methods

The material is the same as that used in the first paper of this series. Data on the clinical aspects of the 20 cultivated tumors, their histologic structure, and the methods utilized in their study are sufficiently detailed in the previous paper (*1*).

Silver impregnations of Río Hortega and Bodian have presented us with the pictures of connective fibrils that form the basis for the following descriptions.

Results

The staining of argyrophilic, intercellular fibrils in sections corresponded with the descriptions published by Río Hortega (*4*, *5*), Alpers, Yaskin and Grant (*6*), Costero (*7*), Río Hortega, Prado and Polak (*8*), and Prado and Oribe (*9*), as components of the connective-tissue stroma of meningiomas. Therefore, it need only be mentioned here that in the majority of our cases the reticulum network was limited to bundles accompanying the blood vessels (fig. 1) leaving large groups of cells surrounded by fibers but completely devoid of them. The three nodular, psammoma-type meningiomas showed many more argyrophilic fibers but without definite organization, as can be seen in figure 2. Here the cells were not stained, but closed networks corresponding to some psammoma bodies could be seen. In the two angiomatous meningiomas of this series there was a greater development of the precollagenous reticulum (fig. 3) from a delicate but well-defined pericapillary adventitia. In cases 4, 6, 8, 12, 13, 17 and 18 there were a considerable number of collagenous bundles, and in four of these cases the bundles were arranged in large plaques that appeared to be better related to the adjacent dura than to the neoplastic cells. However, in case 17 there were areas where collagenous bundles were evenly distributed (fig. 4). The explanted meningosarcoma showed the characteristic reticulum of delicate filaments and unicellular network reproduced in figure 5.

Ever since the memorable paper by Mallory (*10*), all authors working on the histologic structure of meningiomas have described or mentioned the intracellular fibrils, which can be demonstrated in practically all cases provided the material is in good condition (fig. 6). Either with aniline dyes or with silver impregnations, they have been studied by Alpers, Yaskin and Grant (*6*), Bailey (*11*), Río Hortega (*4*, *5*), Río Hortega, Prado and Polak (*8*), and Cid (*12*) in histologic sections, and by Bland

and Russell (*13*) and Murray (*14*) in tissue culture. We have observed them in large numbers in cases 4, 7, 8 and 9. They were found in isolated areas in cases 3, 5 and 12, but we failed to stain them in cases 2, 6, 11 and 20.

Intercellular fibers appearing in meningiomas in culture very often show morphologic features corresponding to the reticular fibers of loose connective tissue, particularly to those altered by proliferative inflammation. They are simple filaments of no more than 1 micron in thickness and anastomosed in a tridimensional network of very irregular threads. They have an intense affinity for the silver carbonate of Río Hortega's method, the capacity to reduce Bielschowsky's solution under certain conditions, are negative to Bodian's impregnation, and are weakly and inconstantly acidophilic. *In vitro* they show even more variation than in histologic sections and, therefore, cannot be described according to a single pattern. There are at least two main types: 1) Fibers, strictly speaking, disposed variably according to the time elapsed since the beginning of their development and also according to the density of the solid phase of the culture, and 2) cellular, filamentous processes of reticular fibrocytes.

Intercellular Fibers

Fibers properly speaking are, at the beginning, very light and thin. As shown by Maximow (*15*, *16*), McKinney (*17*), and Levi (*18*, *19*), the first to reduce silver carbonate are in the immediate neighborhood of fibroblasts associated in well-defined groups (fig. 7). They rapidly extend in parallel directions and are in intimate contact with the main processes of the corresponding fibroblasts. These cells are thus so tightly enveloped by the fibers that it is extremely difficult to decide, from the study of photographs, the exact nature of the relationship that no doubt exists between fibers and cells during connective-tissue fibrogenesis.

In areas where fibers are still unstained, fibroblasts appear as anastomosed cells of simple shape. Generally these are fusiform or triangular. Often these fibroblasts contain fine argyrophilic granulations in their somatic cytoplasm, which we have been unable to identify with certainty and which sometimes take up aniline dyes. Argyrophilic granulations of fibroblasts are usually more numerous in the periphery of the cellular body than around the nucleus and frequently appear elongated as small rods (fig. 8).

The first intercellular fibers to appear for some time may form short and poorly arranged fascicles but soon anastomose profusely with each other. When they separate after longer incubation they extend into highly complex, irregular networks. The density of the network varies with the consistency of the solid phase of the culture medium. In areas where the plasma clot preserves all its natural consistency, fibers first develop a feltwork of very thin threads and nets, which are so tight that they form wide membranous plaques instead of fibrillary networks surrounding the fibroblasts (fig. 9). It is possible that the intercellular feltworks of precollagenous fiber were the nets considered by Baitsell (*20*) as fibrin because

both have some morphologic similarities. These dense portions of the pericellular feltworks become gradually better individualized fibers. On the other hand, similar fibers appear immediately next to the fibroblasts in areas where the culture medium is less dense. When the explanted fragment retracts and a halo without solid phase appears around it, argyrophilic fibers formed in the space are thicker, relatively widely separated from each other, and more permanently closely associated with the fibroblasts (fig. 10).

As little as 48 hours after the appearance of the first argyrophilic fibers, in favorable cases the newly formed network can be as complex as the one seen in figure 11. Here the two main trends of ultimate fiber development are already discernible: *a*) some of them will remain anastomosed to form a network and even if some of their elements show a greater thickness than others they will not attain sufficient individuality as to alter the shape of the group; *b*) others will increase in thickness, will dissociate into very delicate filaments permanently fused into fascicles, will simplify their anastomoses and become more acidophilic until they are comparable to collagenous bundles.

In our explants the majority of the intercellular, neoformed fibers maintained as argyrophilic networks became homogeneous with time and adapted the shape of their threads to the enclosed cells. However, not all intercellular fibers stainable with silver carbonate in meningioma cultures were new formations. Some originated through the elongation of reticular fibers within the explant. In most cases, differentiation between these types was easily achieved, since young fibers were relatively thin, pale, and poorly defined when compared to the pre-existing ones. The latter formed better individualized networks, reached the area of growth more slowly than the cells and soon lost their cellular relationship. This brought about a very characteristic dissociation between cells and reticulum at the level of the explant which was even more distinct in contrast to the outgrowth picture where fibers and cells made up a coherent whole. After the third week in culture most of the pre-existing reticular fibers had grown enough to intermingle with the newly formed ones in the outgrowth. At this time reticular fibers as dense and as well organized as the ones preexisting in the explant could form away from the explant in the culture medium through the action of migrated fibroblasts (fig. 12).

In contrast to reticular fibers completely developed collagenous bundles, which are frequently present in the explanted fragments of tissue, never showed signs of growth or of any reactional change. They remained permanently unaltered in short-term cultures. This marks a definite difference between reticular, precollagenous and predominately argyrophilic fibers on one hand, and fasciculated, characteristically acidophilic collagenous bundles on the other.

Reticular Fibrocytes

Filamentous cellular processes are less easily discovered in cultures. They may develop in any part of the outgrowth, included and hidden

within the mass of fibroblasts. The cells from which the fibers originate then can be identified only if they take the dyes with greater intensity than usual and even so the filamentous processes cannot be distinguished from the fibers of the intercellular reticulum. Therefore, it is better to study them in those places where they develop alone, independent from fibroblasts and from intercellular fibers. This often happens in the distal limits of outgrowth and in some clear halos produced by retraction of the explant. Under these conditions the cellular processes in many places show morphologic and tinctorial features identical with those of the fibers developed between the cells. They fuse and anastomose freely with these fibers (figs. 13 to 16).

As reticular fibrocytes we define cells with filamentous processes in tissue culture since we believe they are static elements comparable to the corneal cells of the eyeball, the cells of tendons and, in general, to the stellate and anastomosed fibrocytes found in places where active fibrogenesis is completed. The length of the processes given off by reticular fibrocytes is greater and their anastomoses clearer because of the peculiar physicochemical conditions that occur during growth *in vitro*. The reciprocal anastomoses among filamentous processes and between the latter and the intercellular fibers constitute the most important data for the inclusion of fibrocytes within the connective-tissue pattern. The stellate elements of nervous tissue archetype found in cultures show free ends to their processes, among other morphologic features of their own.

Reticular fibrocytes were easily identified in our cultures. They were found in 75 percent of the preparations. They occurred in 2 cases with maximal intensity, in 9 with moderate intensity, and in 5 in a smaller proportion. Almost always they presented a complex polymorphism, as can be seen in figures 13 to 16. A careful analysis of slides stained with Río Hortega's method showed that where reticular fibers surrounded fibrocytes there were two superimposed plexuses, one cellular and the other fibrillar (fig. 13). When intercellular fibers develop it is not difficult to separate such fibers from the cells since most of the latter show a large cellular body size and are devoid of granulations or fibrils (fig. 14). Careful study shows that not all fibers lying among the cells are entirely independent from them. Some of the fibers behave as argyrophilic cellular prolongations.

However, in order to be certain that the filamentous processes originating in reticular fibrocytes contribute to the formation of the intercellular reticulum, it is useful to study such areas where only stellate cells are found. This is not difficult in cultures of more than 5 weeks of age. Either in the halo surrounding the explant or in the distal portion of the outgrowth (figs. 15 and 16), reticular fibrocytes can be seen with prolongations, some of which show an initial ribbonlike portion with a structure identical to the cytoplasm of origin. These processes immediately change to argyrophilic filaments anastomosing with each other and, when present, with reticular fibers developing among the cells. In some places the filaments become longer and wider, and branch into acidophilic fascicles

with a tendency to hyalinization as in collagenous bundles. This can be seen in several places in figures 15 and 16. The development in cultured meningiomas of fibrocytes giving origin to processes composed of three successive portions (ribbonlike protocollagen, reticulate precollagen and fasciculated collagen) reproduces *in vitro* a fibrocellular structure comparable to the reticular network of the spleen and definitely relates the intercellular connective fibers to fibroblasts.

According to the old conception of French histologists, we consider the fibrocytes as inactive fibroblasts, *i.e.*, they are cells no longer engaged in fiber formation. Thus we found fibrocytes only in old cultures where cellular migration and fibrogenesis had stopped or when the connective cells had adopted a fixed position, such as becoming attached to the surface of the cover slide. The most common circumstance for the transformation of active or true fibroblasts into static fibrocytes follows after marked increase in the volume of the whole cell. At this time the cytoplasm of the fibroblast is extended on a large surface and appears laminar and hyaline when stained with aniline dyes, and the nucleus reaches its greatest size. After this stage, the predominant morphologic type has a relatively small and chromophilic cellular body with most of the cytoplasm distributed in many processes more or less hidden among neighboring cells. The nucleus is rounded, kidney-shaped, or slightly lobulated. Sometimes there are two or three nuclei, always with several polymorphic nucleoli. Argyrophilia of such fibrocytes is greater at the level of the processes and covers a wide range of intensity as can be seen in the various elements shown in figures 17 to 20.

Intracytoplasmic Fibrils

The argyrophilia of fibrocytes can extend from the prolongations to the cellular body. This is due to the presence of fibrils in the perikaryon. Intracytoplasmic fibrils of fibrocytes often are subtle, pale, abundant and fasciculated such as the "fibroglia fibrils" of Mallory (*10*) (fig. 17). In some cells these fibrils are better defined, reticulated and highly argyrophilic—resembling the "inofibrils" of Tello (*21*) (fig. 18). Occasionally these two types are mixed with other fibrils which appear as thick lines stained black by silver, corresponding to wide fibrillary fascicles extending along opposite cellular processes (fig. 19) and showing incipient hyalinization. As Mallory observed, intracytoplasmic fibrils are as thick as the cellular processes along whose course they travel. They also traverse the somatic portion of the fibrocyte as in the case of the neuroglia fibers within the astrocytes. Occasionally the filamentous processes become fascicles of threads filling the cellular body resembling tonofibrils. Finally, reticular fibrocytes can also be found in which the extremely argyrophilic filamentous processes are decomposed into very light fibrils, which are soon lost within the cytoplasm of the perikaryon (fig. 20).

It would seem that the polymorphism of the intracytoplasmic fibrils found in our cultures of meningioma tissue were adequately illustrated. However, we only achieve a satisfactory knowledge of the meaning of the

filaments within the fibrocytes when we study cells in direct contact with the surface of the cover slide. The thinness of the cellular body is then highly favorable for staining the most delicate threads. In figure 21 a fibrocyte is shown which has a reticulated filamentous network; the fibrils leave a very narrow and regular halo surrounding the nucleus and fall in short groups into the culture medium giving the impression that there are no precise cellular limits. Only on the upper part of the cell do the filaments fuse into a well-outlined hyaline mass and continue into a highly argyrophilic process. Figure 22 shows a reticular fibrocyte of odd morphology, probably due to the fact that it adhered to the cover slide. All cytoplasmic fibrils are almost parallel to each other and follow the course of the innumerable and gracefully anastomosed prolongations that open as a fan from the perikaryon. Filamentous processes, similar to the ones from the preceding cell but more argyrophilic and similar to intercellular precollagenous fibers, are preserved in the fibrocyte reproduced in figure 23. The description of intracellular fibrils is completed with figure 24, showing a fibrocyte containing interlaced, curious fascicles whose cytoplasmic network is condensed into filamentous processes.

Intracellular Connective Fibrogenesis

We believe it has been shown: 1) that at least in *in vitro* experiments, active fibroblasts arising through nomicoplasia from meningioma cells regularly become static, stellate fibrocytes; 2) that processes originating from these fibrocytes in many places acquire histochemical features similar to those of the reticular fibers; and 3) that such processes are continuous with collagenous bundles at their distal end and with intracytoplasmic fibrils at their proximal end. However, the basic problem of connective fibrogenesis is still obscure. For example, we have ignored the relationship linking fibroblasts with collagenous bundles.

In an attempt to demonstrate the morphologic process of *in vitro* transformation of reticular fibers into collagenous bundles, we used aniline blue and van Gieson's picrofuchsin for staining collagen. Both stains were used alone or as a counterstain after silver impregnation. In this way, we found collagenous bundles within connective-tissue cells. We immediately reviewed all our slides in an attempt to interpret such a striking finding and believe we have enough data to reconstruct all phases of intracellular fibrogenesis. The process seems to develop along the following lines.

The first morphologic sign of specific fibroblastic activity is inconstant and is based on the appearance of granulations, a few of which are still present in figure 25. It is possible that these granulations play some role in the genesis of fibrils and perhaps Meves (*22*) is referring to them when he says that connective fibers arise from mitochondria. This may also apply to Cajal (*21*) in the development of his theory of inobiones. Immediately following the formation of granules, the cytoplasm becomes condensed in poorly individualized filaments, which are usually located in the periphery of the cellular body, as can be seen in the same figure, or in

the processes when present. Soon such fibrils reach sufficient autonomy as to make their course through the perikaryon clearly visible. Here small vacuoles develop, which probably replace the granulations (fig. 26). Infrequently the fibrils go a step beyond in their development and push the nucleus to one side. More often they soon leave the cytoplasm, separating from the cell in the manner described by Schwann and Boll [cit. Mall (*23*, *24*)] and Flemming (*25*, *26*) more than a century ago (fig. 27). During these first stages the fibrils arising within the cytoplasm can be stained with both Bodian's and Jacobson's methods, but it is necessary to look very carefully for them. They are very fine and have a very weak affinity for dyes compared with the surrounding medium. The transient nature of such primordial structures, as well as the fact that they are very probably reversible in character, all contribute to making them escape the attention of observers.

From this stage of development, however, the fibrils become easily stainable with some silver impregnations, especially with the ammoniacal silver carbonate of Río Hortega. A few fibers are retained for a short time within the cytoplasm of some fibroblasts and constitute Tello's inofibrils. Other fibers, in no greater number, can still be seen in the exoplasm of fibroblasts as shown in figure 28. Commonly, however, when becoming extracellular the vast majority of fibrils change their affinity for the different stains and acquire the histochemical properties of reticular fibers. Then they surround and embrace the cells in such a complex manner that it is extremely difficult to determine their preceding relations with them.

Fortunately, the protocollagenic nature of intracytoplasmic fibrils can manifest itself within the cells. No doubt this happens only in exceptional cases. A very favorable one of these is found in the case of meningiomas in culture. The broken up perikaryon of the cell, reproduced in figure 29, is also occupied by a meshwork of material giving the reactions for collagen, originating from argyrophilic fibers that still can be seen running along the filamentous processes of the cell. Complete collagenous transformation of the intracytoplasmic network can be clearly seen in the cell in figure 30. Finally, the hyaline areas, which have been described in the filaments of the cells photographed in figures 19 and 28, also represent the collagenous transformation of the intracytoplasmic networks of connective-tissue cells.

We are now in a better position to understand the meaning of the intracytoplasmic fibrils of fibrocytes and other connective-tissue cells. In the same manner as we have demonstrated that intracytoplasmic fibrils (protocollagen) are capable of becoming argyrophilic (precollagen) and acidophilic (collagen) fibers without leaving the cells where they are formed, fibroblasts may preserve their intracytoplasmic fibrils when their specific functional activity is in abeyance or is lost and they become fibrocytes. Furthermore, all connective-tissue cells may contain intracytoplasmic fibrils without forming intercellular fibers, *i.e.*, without becoming fibroblasts. In other words, the specific activity of fibroblasts is the

formation of intercellular filamentous connective substance. Despite the fact that they are protocollagenic in texture, intracytoplasmic fibrils characterize only the connective-tissue nature of the cells containing them.

According to that hypothesis, fibroglia fibrils, inofibrils and tonoplasms of connective-tissue cells represent the static prophase of desmofibrogenesis. Thus, morphologic differences that have served as a basis for such varied names are not, in our opinion, adequate justification to use them as antonyms, suggesting different structure. On the contrary, the coincidence in the same cultures of the wide variety of morphologic pictures that we have described above indicates that the polymorphism of the intracytoplasmic fibrils of connective-tissue cells must be a consequence of secondary, transitory and extrinsic factors.

We consider fibroglia fibrils, inofibrils, tension fibrils and related fiber structures as varieties of *desmofibrils*. This is a histologic name that we suggest in analogy with epithelio-, neuro- and gliofibrils, the corresponding structures in epithelial and nervous tissues which never become detached from the cells nor tend to become collagenous substance. Thus, desmofibrils are intracytoplasmic protocollagenous fibrils which may appear in all connective-tissue cells when going through nomicoplasia, but these cells should not be considered as true fibroblasts until the desmofibrils are transformed into intercellular precollagenous or collagenous fibers.

Discussion

The time when the term "fibroblasts" was used in a negative sense as a designation for those connective-tissue cells which were so poorly differentiated that no other name could reasonably be employed should be considered as past. Similarly, vascular endothelia, meningeal endothelia, mesothelia and fibrocytes, frequently considered as special kinds of fibroblasts, are apparently similar but actually are different cellular species. In tissue culture where differentiation is still less developed than in ordinary histologic sections, the word fibroblast is even more abused with a vague generic meaning of "mesenchyme-like cell." However, we do not believe that such loose terminology is justified. When we speak here of fibroblasts, we are referring, in a more restricted way, concretely and exclusively to those connective elements coexisting in intimate relationship with intercellular fibrils in the process of formation.

It seems certain that fibroblasts, developed through nomicoplasia in our cultures of meningiomas, actively elaborated collagenous bundles. This fibrogenesis starts as thin, transient, intracytoplasmic, protocollagenous desmofibrils, the vast majority of which soon become reticular precollagenous fibers. Later a variable amount of the reticular fibers change into collagenous bundles. Although, as a general rule, these last two phases of fibrogenesis take place outside of the cells, sometimes they may proceed without leaving the fibroblasts, a circumstance showing the protocollagenous nature of intracytoplasmic desmofibrils. Furthermore, when fibrogenesis slows down or stops, active fibroblasts become static

fibrocytes giving origin to long, filamentous processes often made up of three successive portions: ribbonlike protoplasm, filamentous precollagen and fasciculated collagen. Moreover, fibrocytes may preserve desmofibrils with the morphology of fibroglia fibrils, inofibrils, tonoplasms and many other types—all without any known special significance. Desmofibrils may also be present in other varieties of connective-tissue cells, as in vascular and meningeal endothelia. This fact means that the fibroblastic system is formed by fibroblasts as active members, plus fibrocytes and endothelial linings as associate members.

Desmofibrils behave in fibroblasts as unstable, perhaps reversible structures, and in fibrocytes and endothelia as permanent, static filaments. Reticular fibers grow rapidly in cultures and in many pathologic processes they adapt to intercellular spaces and form a stable network. Finally, collagenous bundles play a passive role without showing any sign of adaptation or reaction to abnormal or experimental conditions and tend to hyalinization and retraction as colloidal gels. All of these three successive varieties of fibers may remain indefinitely as such in the normal organism.

These differences in themselves are sufficient to meet the modern tendency to consider all connective fibers as collagen and equivalents. It is true that until now, chemists [Siegfried (*27*, *28*), Foot (*29*), Lowry, Gilligan and Katersk (*30*)], workers with X-ray-diffraction patterns [bibliography in Gross and Schmitt (*31*)] and experts in electronic microscopy [bibliography in Vanamee and Porter (*32*)] have been unable to find conspicuous differences between desmofibrils, reticular fibers and collagenous bundles. However, such lack of difference has never been interpreted by the specialists as identity, nor has it been universally accepted. On the contrary, morphologists cannot consider structures so well defined histologically and histochemically as the ones in question as being equal. It is necessary to distinguish the three varieties of filaments with the terms desmofibrils, reticular fibers and collagenous bundles. Otherwise, considering their most distinguishing physico-chemical properties, they may be designated as protocollagenous fibrils, precollagenous fibers and collagenous bundles, respectively.

The key problem, however, is to find out whether intracellular fibrogenesis is, or is not, specific for meningioma cells. The explantation of the other tissues, the daily practice of pathology and careful study of classical papers published on the subject has led us to presume that connective fibrogenesis constitutes a phenomenon present in a similar form in the most diverse conditions. The bibliography on the histogenesis of connective fibers is very abundant. The most relevant papers have been reviewed by Foot (*29*), Maximow and Bloom (*16*), McKinney (*17*), Jordan (*33*) and Fischer (*34*). Modern concepts concerning connective tissue, especially those referring to ground substance and to the physico-chemical composition of collagen, can be found in the recent books edited by Ragan (*2*).

In view of the results of our work and the data accumulated in the literature, we believe that a *fibroblastic system* exists in definite areas of the organism which is somewhat opposed to the reticuloendothelial system, although keeping a certain relationship with it. Thus, in the same way as the reticuloendothelial system is polyblastic (a source of the differentiation for all connective-tissue elements and important in immunity responses), the fibroblastic system is nomicoplastic, constitutes the final state during legitimate involution of the same elements, and proves the morphologic substratum for hypersensitivity reactions. Since the development of such an important problem is beyond the subject of this paper, it will be the theme of later publications.

Summary

1) Fibroblasts, originated through nomicoplasia in tissue culture, elaborate protocollagenous, intracytoplasmic desmofibrils. The majority of these desmofibrils in culture soon become precollagenous reticular fibers and a small proportion of the latter acquire the histochemical properties of collagenous bundles. Collagen substance, therefore, is believed to be of intracellular origin, at least under the conditions described.

2) In some nomicoplastic tumor cells fibrogenesis is slowed down or stopped. The resulting inactive cells give origin to processes which are sometimes composed of three successive portions: ribbonlike protoplasmic, cylindroid argyrophilic and fasciculated acidophilic. These prolongations are mixed and anastomosed with the intercellular reticulum. Often other similar cells preserve their desmofibrils; *i.e.*, they are cells with fibrils, or fibrocytes. In addition, all connective-tissue cells, as a manifestation of their connective-tissue nature, may contain desmofibrils without forming intercellular fibers.

3) Fibroglia fibrils, inofibrils and tonoplasms or tension fibrils correspond to three of the morphologic aspects that desmofibrils may present within connective-tissue cells. Only fibroblasts are capable of transforming desmofibrils into collagenous bundles.

4) Desmofibrils, reticular fibers and collagenous bundles, representing successive stages of the same process of connective fibrogenesis, may be preserved in normal tissue as permanent structures and show properties conferring outstanding individuality to each one of them. Desmofibrils are intracellular and reversible, reticular fibers possess extracellular autonomous growth, and collagenous bundles are inert structures.

5) Because they are endothelial in texture, meningioma cells contain intracytoplasmic desmofibrils and form cords devoid of intercellular precollagenous fibers or collagenous bundles. Only when they manifest nomicoplasia, both *in vivo* and *in vitro*, do meningioma cells become active fibroblasts. A similar secondary fibroblastic activity is present in all kinds of connective-tissue tumors. This fact sharply differentiates meningiomas from fibroblastomas, the latter being the only connective-tissue tumor in which neoplastic cells are primarily fibroblasts.

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PLATE 87

Main forms of reticular network in meningiomas impregnated with Río Hortega's silver carbonate

FIGURE 1.—Most frequently found picture in typical meningiomas; the connective reticulum accompanies the blood vessels, leaving large groups of tumor cells devoid of fibers. × 70

FIGURE 2.—Psammomatous meningioma showing abundant argyrophilic fibers without definite organization. × 45

FIGURE 3.—Angiomatous meningioma with greater development of the precollagenous reticulum from a well-defined pericapillary adventitia. × 70

FIGURE 4.—Collagenous bundles developed in case 17 and evenly arranged among the neoplastic cells. × 45

FIGURE 5.—Reticulum made up of delicate filaments which form unicellular networks characteristic of meningosarcomas. × 110

FIGURE 6.—Intracytoplasmic filaments (desmofibrils) impregnated with silver in the same case of meningosarcoma. × 110

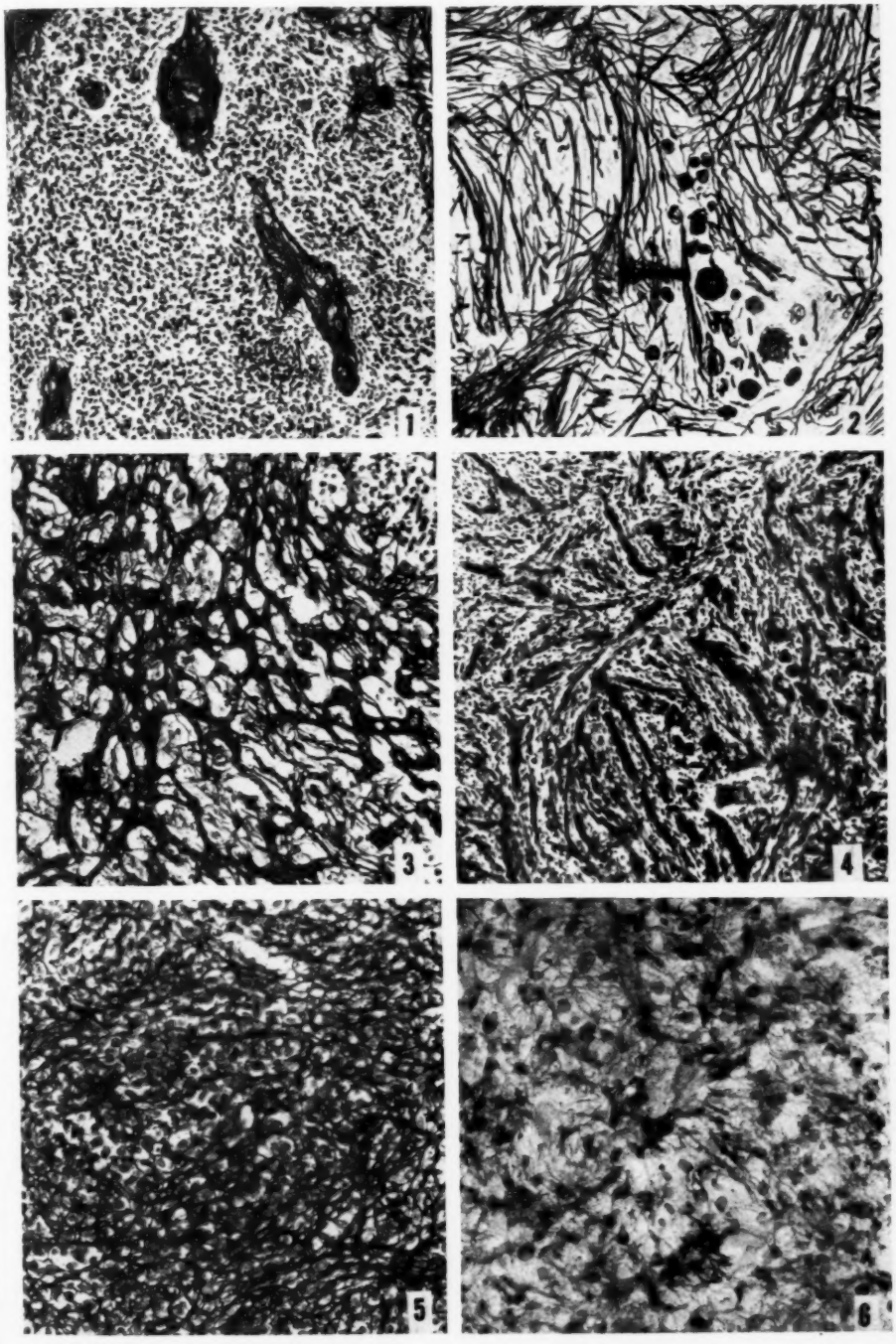

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PLATE 88

Intercellular fibers developed in cultures of meningiomas after nomicoplastic transformation of tumor cells into fibroblasts. Case 10. Río Hortega's.

FIGURE 7.—Extremely delicate networks, the first to reduce silver carbonate, enveloping groups of fibroblasts. (S.XXX-F.3.) 27-day culture. × 110

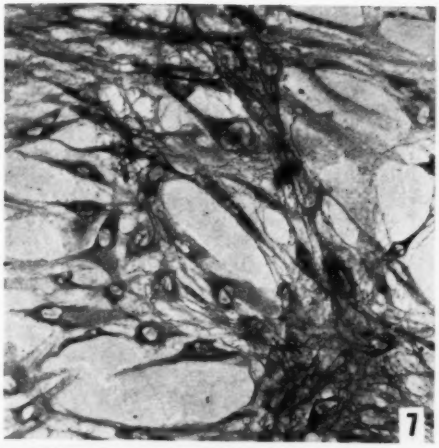
FIGURE 8.—Argyrophilic granulations frequently present within fibroblasts during the initiation of connective fibrogenesis. (S.XXX-F.3.) 27-day culture. × 220

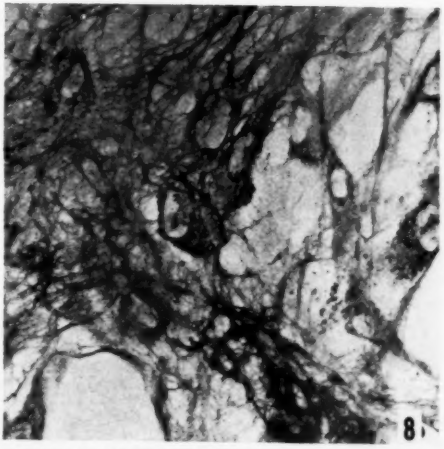
FIGURE 9.—Feltwork of very fine precollagenous threads expanded into wide membranous plaques at the beginning of connective fibrogenesis *in vitro*. (S.XXVI-F.2.) 21-day culture. × 220

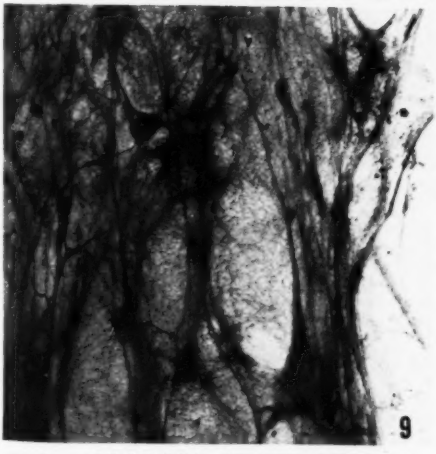
FIGURE 10.—Thicker argyrophilic fibers developed in the halo surrounding the explant in close association with the fibroblasts. (S.XXVI-F.2.) 21-day culture. × 220

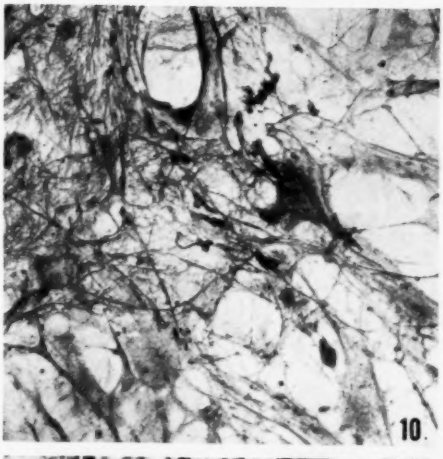
FIGURE 11.—Intercellular precollagenous fibers which appeared in the outgrowth of meningioma cultures a few hours after the appearance of the first fibroblasts. (S.XXVI-F.2.) 21-day culture. × 220

FIGURE 12.—Complete development of the intercellular networks of argyrophilic fibers. (S.XXVI–F.2.). 21-day culture. × 220









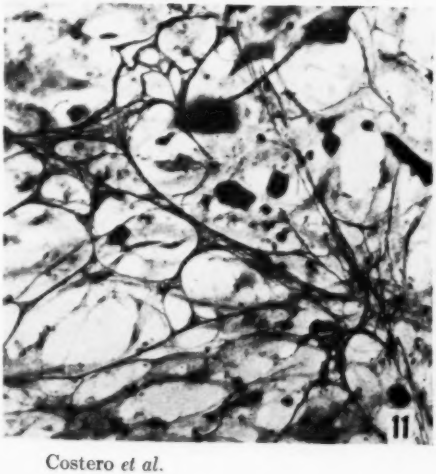


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PLATE 89

Diverse pictures of reticular fibrocytes. Case 10. (S.XXVI-F.2.) 21-day culture. Río Hortega's. × 220

FIGURE 13.—Cellular and fibrillary plexuses, the former gray in color, the latter black for silver impregnation.

FIGURE 14.—Fibrocytes showing their large cellular bodies devoid of granulations or fibrils. Surrounding them are precollagenous fibers.

FIGURE 15.—Argyrophilic filamentous prolongations of fibrocytes showing anastomoses everywhere with the intercellular precollagenous reticulum.

FIGURE 16.—Reticular fibrocyte that gives off a prolongation with three characteristic successive portions: ribbonlike protoplasmic, threadlike precollagenous and fasciculated collagenous.

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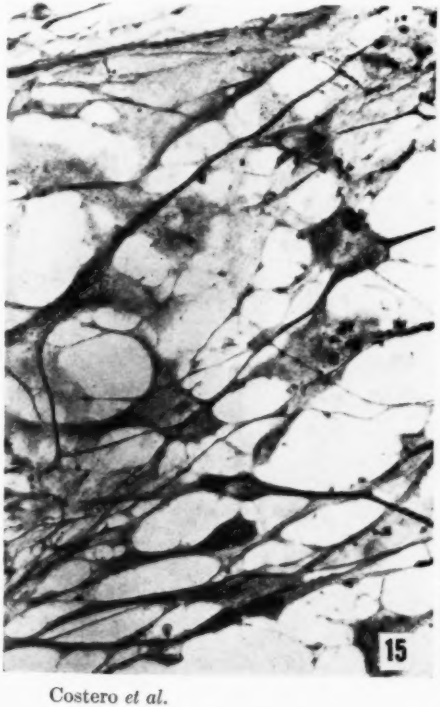
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PLATE 90

Appearance of intracytoplasmic desmofibrils in fibrocytes developed in old cultures. Case 4. (S.XII–Fs.1,2.) 49-day culture. Bodian's.

FIGURE 17.—Subtle, pale, abundant and fasciculated desmofibrils (fibroglial type) within a fibrocyte. × 220

FIGURE 18.—Better defined, reticulated and highly argyrophilic desmofibrils (inofibrillar type) within another fibrocyte. × 440

FIGURE 19.—Wide fascicles of desmofibrils extended along opposite cellular processes within another fibrocyte. × 110

FIGURE 20.—Desmofibrils traveling along the processes of a fibrocyte and filling the cellular body (tonofibrillar type). × 440

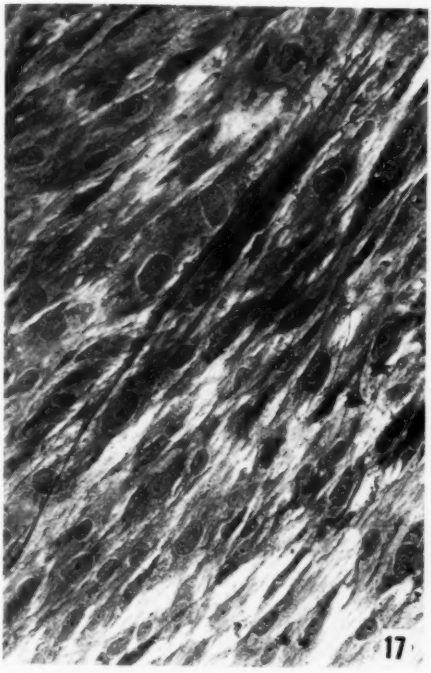
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PLATE 91

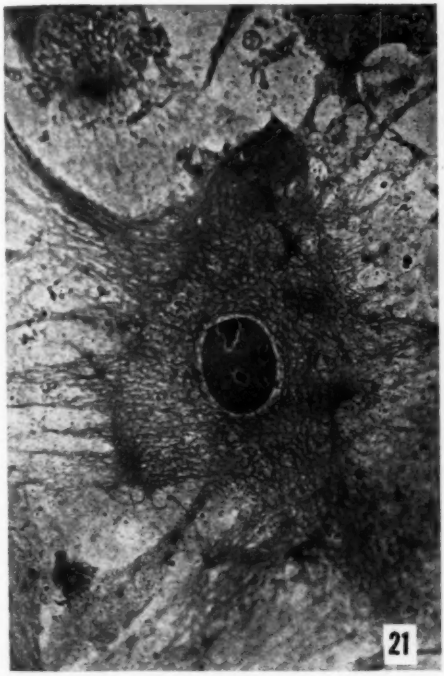
Demonstration of the extreme polymorphism of desmofibrils when fibrocytes were adapted to the cover slide. Case 2. (S.II-Fs.3,4.) 30-day culture.

FIGURE 21.—Fibrocyte with recticulated desmofibrils, perinuclear halo and diffuse cellular limits. × 440

FIGURE 22.—Parallel arrangement of the desmofibrils inside the perikaryon and processes of a fibrocyte with an odd morphology. × 440

FIGURE 23.—Desmofibrils in this cell became intensively argyrophilic and filamentous processes exhibited a reticular-precollagenous texture. × 220

FIGURE 24.—Fibrocyte containing interlaced fascicles of desmofibrils growing into the filamentous processes. × 220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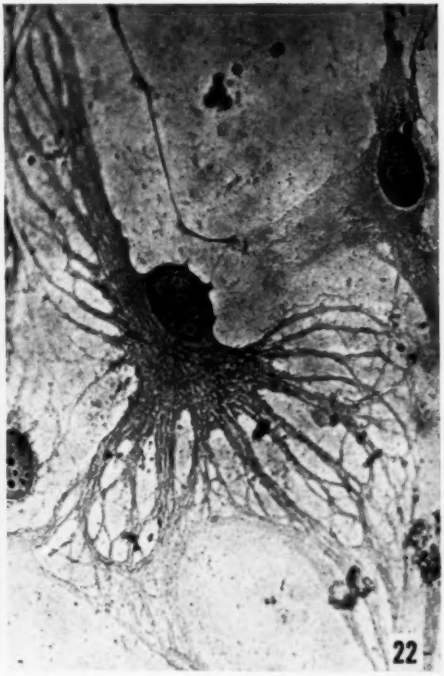
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PLATE 92

Main steps of intracellular formation of collagen in cultures of meningiomas. Case 4. Bodian's. × *440*

FIGURE 25.—Initial condensation of the cytoplasm into poorly individualized filaments, preferentially located in the periphery of the perikaryon and in the processes. (S.XII-F.2.) 49-day culture.

FIGURE 26.—The filaments reach sufficient autonomy as to make their course through the perikaryon clearly visible. (S.XII-F.1.) 49-day culture.

FIGURE 27.—Completely formed desmofibrils leaving the cellular body of the fibroblasts. Note intra- and extra-cellular fibrils in intimate relationship with each other. (S.XII-F.1.) 49-day culture.

FIGURE 28.—Intense argyrophilic desmofibrils can still be seen in the exoplasm of fibroblasts. (S.XII-F.2.) 49-day culture.

FIGURE 29.—The broken up perikaryon of this cell is also occupied by a meshwork of material with the reactions of collagen. (S.III-F.1.) 20-day culture. Counterstained with van Gieson's picrofuchsin.

FIGURE 30.—Complete collagenous transformation of desmofibrils; only some of them leave the fibroblast. (S.III-F.1.) 20-day culture. Counterstained with van Gieson's picrofuchsin.

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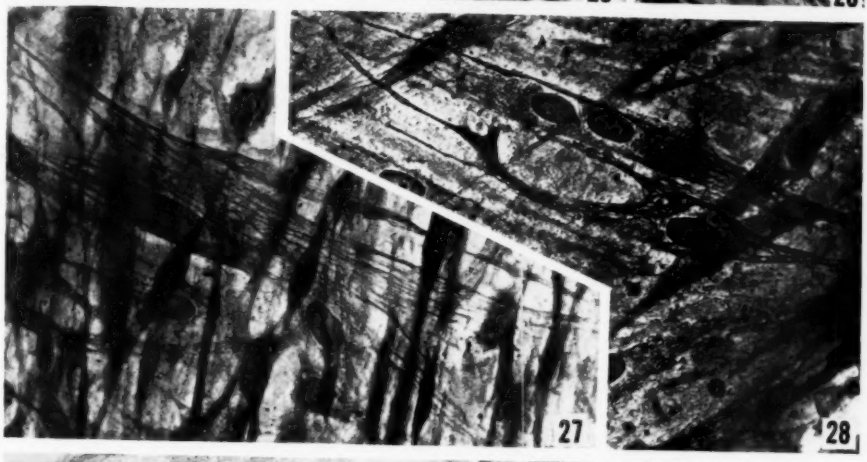
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Intraperitoneal Transplantation of the Yoshida Ascites Sarcoma and the Ascites Hepatoma to Various American Strains of Rats[1]

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In December 1952, the Yoshida sarcoma and two strains of ascites hepatoma were brought to this laboratory and have been systematically transplanted to various American rat strains and to one strain secured from Japan. This paper is concerned with their growth characteristics and reports the successful establishment of these ascites tumors in rat strains obtainable in this country.

I. The Yoshida Ascites Sarcoma

The Yoshida sarcoma, an ascites tumor, was discovered in 1943 by Yoshida and his co-workers in one of 20 rats fed with *o*-aminoazotoluene and also painted on the skin of the back with potassium arsenite solution (*1*). Successive transplantations of this tumor have been carried out in Yoshida's laboratory and several reports have appeared with regard to its rate of growth (*2–5*). Although most of the animals employed in Japan were not of an inbred strain, the successful transplantation rate averaged more than 91 percent through 322 generations (*6*).

Materials and Methods

A strain of Japanese rats[4] and eleven strains of American rats, both inbred and noninbred were used in this study as follows:

Noninbred	*Inbred*	*F_1 hybrids*
Japanese	Marshall 520*	[Marshall 520 × (A × C9935)] F_1†
Sprague-Dawley	Buffalo	
Holtzman	Wistar	
NIH black	Fischer	
	O'Grady	
	Albany	
	Osborne-Mendel	

[1] Received for publication September 20, 1954.
[2] Postdoctorate NCI research fellow.
[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.
[4] A noninbred strain obtained from Dr. Yoshida's laboratory in Tokyo. This strain, and all others used in these experiments, was obtained from the colonies raised at the National Institutes of Health.
*This strain is referred to in the text and tables as M 520.
†This strain is designated in the text and tables as M 520 × AC.

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The donor animals received from Japan carried the Yoshida sarcoma in the 395th serial transplant generation.

Transplantation of the Yoshida sarcoma was performed weekly, according to the method of Yoshida (7), *i.e.*, the abdominal wall was punctured with a sharp-pointed, sterile glass pipette (drawn from 4 mm. outside diameter glass tubing). In this way, a quantity of tumor ascites sufficient for observation or transplantation was easily obtained. Typically, 0.02 to 0.1 cc. of tumor ascitic fluid containing about 20 to 100 million tumor cells was transplanted to each of a group of rats weighing 50 to 150 grams. After inoculation, fluid was withdrawn each day from the peritoneal cavity, and proliferation of tumor cells was followed in fresh and Giemsa-stained smears until the death of the animal bearing the tumor. Animals in which the inoculated tumor cells did not proliferate at all, or proliferated very well initially but later decreased in number and disappeared, were examined for 1 to 3 months. Thereafter, all these animals were sacrificed for pathologic study.

Results

A) *Proliferation and spontaneous regression of tumor cells.*—During a period of 16 months, serial intraperitoneal transplantation of the Yoshida sarcoma was maintained for more than 60 generations. The tumor cells proliferated rapidly in eight American strains of rats (M 520, M 520 × AC, Sprague-Dawley, Holtzman, Buffalo, Osborne-Mendel, NIH black, and O'Grady), as well as in the Japanese strain (table 1). Within 2 to 5 days after inoculation the tumor cells reached a state of "nearly pure culture" (7), and a milky ascites formed in the peritoneal cavity. After 8 to 14 days, this fluid became hemorrhagic, thinner, and increased in quantity (5 to 10 cc.).

TABLE 1.—*Results of the intraperitoneal transplantation of the Yoshida ascites sarcoma*

Strain of rats	Initial proliferation of tumor cells*	Number of animals	Tumor takes	
			(*number*)	(*percent*)
Japanese	+++	125	118	94
M 520	+++	295	253	86
M 520 × AC	+++	213	174	82
Sprague-Dawley	+++	104	54	52
Holtzman	+++	87	44	51
Buffalo	+++	51	8	16
Wistar	++	48	4	8
Osborne-Mendel	+++	73	6	8
NIH black	+++	25	2	8
Fischer	+	35	0	0
O'Grady	+++	45	0	0
Albany	+	38	0	0

*+++=Proliferation of tumor cells reached a state of nearly pure culture. ++=Tumor cells proliferated slightly. +=Tumor cells were alive for a few days in the ascites but did not proliferate.

In rats of the Wistar, Fischer, and Albany strains, the tumor cells were recognizable for a few days following inoculation; in some animals

these cells also proliferated slightly. However, after several days these cells showed degenerative changes and soon disappeared. Within 8 to 12 days after inoculation, the tumor cells in some American strains of rats showed spontaneous regression in a manner similar to that described for some Japanese rats (*7*, *2*, *6*). At this time, the tumor cells appeared swollen, and the nucleus and cytoplasm stained only lightly with Giemsa. In addition, the cytoplasm contained numerous vacuoles. Subsequently, these tumor cells in the ascitic fluid decreased in number, showed karyolysis and cytolysis, and finally disappeared. This series of changes was similar in almost all rats where spontaneous regression of tumor cells occurred.

B) *Rate of tumor "takes."*—In this study, the definition of a tumor "take" was based on the progressive growth of tumor cells, increase of tumor ascites in the peritoneal cavity, tumor invasion to various tissues and death of the animal bearing the tumor.

The rate of "takes" following the intraperitoneal transplantation of the Yoshida sarcoma is shown in table 1. The numbers shown in the column marked "take" indicate those rats which died bearing tumor. All such rats (except for one Japanese rat) died within 14 days after inoculation.

Transplantation of the Yoshida ascites sarcoma in the strain of origin during the past 10 years (*6*) has never resulted in 100 percent "takes," even when a sufficient number of rats was taken into consideration. This indicates the existence of a certain number of rats in which tumor cells show spontaneous regression. In American strains of rats a similar situation was observed.

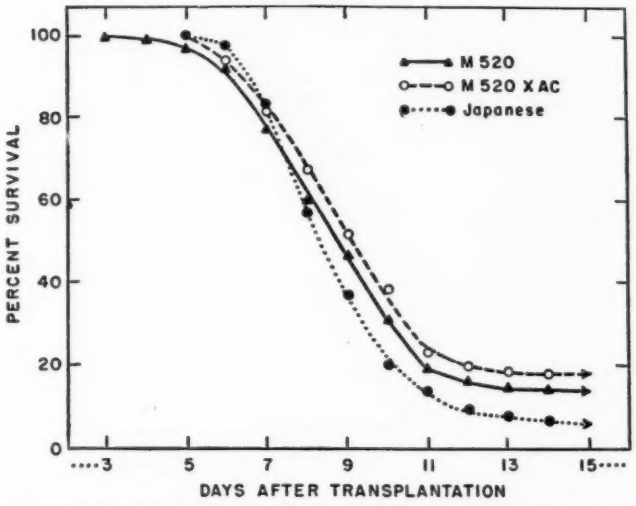
A noteworthy fact is that in some strains of rats (*i.e.*, O'Grady, Fischer, and Albany) no rats died bearing a tumor. That is, all such rats showed complete spontaneous regression after an initial period of cell proliferation, and these strains were considered as nonsusceptible with respect to the Yoshida ascites sarcoma.

C) *Survival of rats transplanted intraperitoneally with the Yoshida ascites sarcoma.*—Survival of rats in which the Yoshida ascites sarcoma "took" are shown in table 2. In M 520 strain rats, 237 of 253 rats which "took" died within 11 days after inoculation. In M 520 × AC, 162 rats out of 174 which "took" also died within 11 days. These results are similar to those obtained in Japanese rats. In text-figure 1, survival curves of transplanted rats of the M 520, M 520 × AC and Japanese strains are presented. Thus, 6 to 11 days constituted the mean survival time. The range of individual variation was small. Even in other strains of rats (Sprague-Dawley, Holtzman, Buffalo, Wistar, Osborne-Mendel), animals with "takes" died within this time, and the average life span for these strains was similar.

D) *Pathologic findings.*—At autopsy of animals with ascites tumors, solid tumor nodules were observed in the omentum, mesentery, retroperitoneal tissue, pelvic cavity and abdominal wall (at the site of puncture). Most of the tumor tissue was very hemorrhagic. In some cases, tumor cells were observed proliferating even in the pleural and pericardial

TABLE 2.—*Survival of rats with intraperitoneal transplants of the Yoshida ascites sarcoma*

Strain of rats	Number of rats dead with tumor													Total number of rats	Average survival
	Days after transplantation														
	4	5	6	7	8	9	10	11	12	13	14	15	21		
Japanese	.....	.....	3	18	33	25	21	9	4	3	1	.....	1	118	9
M 520	2	4	17	42	53	41	43	35	10	4	2	.....	.....	253	9
M 520 × AC	.....	.....	15	22	33	32	28	32	8	3	1	.....	.....	174	9
Sprague-Dawley	.....	.....	3	11	21	14	3	1	1	.....	.....	.....	.....	54	8
Holtzman	.....	.....	.....	12	15	8	6	1	1	1	.....	.....	.....	44	8
Buffalo	.....	.....	2	3	1	1	1	.....	.....	.....	.....	.....	.....	8	8
Osborne-Mendel	.....	.....	.....	1	1	.....	1	2	1	.....	.....	.....	.....	6	10
Wistar	.....	.....	.....	.....	2	1	1	.....	.....	.....	.....	.....	.....	4	9



Text-figure 1.—Survival curve of rats of different strains intraperitoneally transplanted with the Yoshida ascites sarcoma.

fluids. In a few cases, invasion of the mediastinal tissue by the tumor was seen, as had been observed in Japanese rats by Yoshida (*7*, *6*).

Animals that recovered from the tumor, indicating spontaneous regression of tumor cells, were kept under observation for 1 to 3 months after inoculation; they were then sacrificed and examined. In most of them, there was nothing of note except slight adhesion between the omentum and the intestine, liver, spleen or other abdominal organs.

In some animals (mainly in M 520 and M 520 × AC rats) which did not die with a tumor but survived for a long period of time, a large tumor or several tumor nodules were observed at the usual site of invasion (omentum, mesentery, abdominal wall). In this tumor tissue, a large necrotic area was generally observed, but free-tumor cells in the ascitic fluid were no longer present. Yoshida has presented a similar case in which a rat bearing a very slowly growing, solid Yoshida sarcoma tumor survived for 6 months following inoculation (*6*). In this rat, it was implied that tumor cells had proliferated at one time, and then showed incomplete regression. From these data it was presumed that there was a difference among individuals with respect to the resistance they develop against proliferation of tumor cells.

II. The Ascites Hepatoma

Typically, most ascites tumors proliferate in the form of single (free) cells in the peritoneal cavity. In the case of the ascites hepatoma, however, the cells are aggregated to form "islands" which continue to proliferate while floating in the ascitic fluid. Since the establishment of the first ascites hepatoma in 1951 (*8*), various strains have been produced and are

now undergoing serial intraperitoneal transplantation in the laboratories of Yoshida and his associates (*9–12*). In December 1952, two such strains of ascites hepatoma (No. 7974 and No. 130) in the 16th and 57th transplant generation, respectively, were obtained by this laboratory.

Materials and Methods

The ascites hepatomas used in this study were produced by means of two somewhat different procedures. Yoshida *et al.* produced the tumor strain known as ascites hepatoma No. 130 by feeding butter yellow to Japanese rats for a period of 227 days. In addition to the appearance of a solid tumor in the liver, the ascitic fluid in the peritoneal cavity contained a number of free-floating hepatoma islands. This suspension of tumor islands was used for subsequent intraperitoneal transplantations (*9*).

In a similar study, Kaziwara *et al.* fed butter yellow to Japanese rats but produced only a solid tumor in the liver with no appearance of tumor islands in the ascites. A mince of this solid tumor, however, inoculated intraperitoneally, gave rise to an ascites form which was designated as ascites hepatoma No. 7974 (*10*).

With the exception of the NIH black strain, the strains of rats used for the hepatoma investigation were the same as those used in the experiments with the Yoshida sarcoma, as described previously.

Intraperitoneal transplantation of the ascites hepatoma was performed once a week in a manner similar to that described above for the Yoshida sarcoma (*7*). Following inoculation, samples of ascitic fluid containing tumor islands were withdrawn as desired for fresh preparations or for staining with aceto-gentian violet or aceto-orcein.

Results

The results presented in this study represent serial intraperitoneal transplantations through more than 60 generations, covering a period of 16 months.

A) *Growth characteristics and rate of tumor "takes" of the ascites hepatoma.*—Following inoculation, tumor islands of the ascites hepatoma proliferated rapidly, accompanied by a moderate increase of ascitic fluid, and reached a state of nearly pure culture within 3 to 5 days. At this time the ascites typically appeared as a thick, milky white fluid. While the total volume was not large at first, it gradually increased and was accompanied by considerable hemorrhage. Toward the end, the ascites increased in volume from 10 to about 40 cc. and typically appeared thinner but intensely hemorrhagic. This type of proliferation occurred in some strains of American rats such as: M 520, M 520 × AC, Sprague-Dawley, Holtzman, Osborne-Mendel, Wistar, and Albany, as well as in Japanese rats.

In several rats in the above-listed strains, proliferation ceased after a few days, the number of tumor islands decreased, and degeneration occurred. This spontaneous regression occurred usually in 12 to 18 days

after inoculation. In a few cases tumor islands persisted 30 days after inoculation, but thereafter spontaneous regression followed.

In rats of strains O'Grady, Buffalo, and Fischer, growth of the ascites hepatoma was poor. Thus, within a few days after inoculation, tumor islands were present in the ascites and slight proliferation may have occurred but a state of nearly pure culture never was attained.

As previously defined in this communication, a tumor "take" was based upon the following criteria: progressive growth of tumor cells, accumulation of ascitic fluid in the peritoneal cavity, tumor invasion to various tissues, and death of the animal bearing the tumor. Employing this definition, the rates of "takes" in the transplantation of ascites hepatoma No. 7974 and No. 130 are shown in table 3. Although the values in American rats are not so high as in Japanese rats, in both M 520 and M 520 × AC, a sufficient number were successfully transplanted.

TABLE 3.—*Results of the intraperitoneal transplantation of the ascites hepatomas 7974 and 130*

Strain of rats	Initial proliferation of tumor cells*		Number of animals		Number and percent of tumor takes	
	Hep. 7974	Hep. 130	Hep. 7974	Hep. 130	Hep. 7974	Hep. 130
Japanese	+++	+++	86	76	81 (94%)	71 (93%)
M 520	+++	+++	155	142	117 (75%)	99 (70%)
M 520 × AC	+++	+++	127	102	98 (77%)	69 (68%)
Sprague-Dawley	+++	+++	45	43	12 (27%)	11 (26%)
Holtzman	+++	+++	38	17	9 (24%)	4 (24%)
Osborne-Mendel	+++	+++	99	68	12 (12%)	9 (13%)
Wistar	+++	++	37	30	0	0
O'Grady	++	++	35	35	0	0
Fischer	+	+	30	28	0	0
Buffalo	+	++	25	45	0	0
Albany	+++	+++	35	48	0	0

*+++=Proliferation of the hepatoma islands reached a state of "nearly pure culture." ++=The hepatoma islands proliferate slightly. +=The hepatoma islands were alive in the ascites for a few days but did not proliferate.

It is interesting to note that in some strains of rats (Albany, Wistar, O'Grady, Buffalo, and Fischer) no rats died with tumor. The tumor, however, could be carried in some of these strains by serial intraperitoneal transplantation if this was repeated once each week while the tumor islands were still in a state of progressive proliferation. However, such animals never died as a result of the tumor and therefore were considered nonsusceptible with respect to the ascites hepatoma.

B) *Survival time of rats transplanted intraperitoneally with the ascites hepatomas.*—Survival times for rats which "took" with the ascites hepatomas are shown in tables 4 and 5. This distribution covers a relatively long time-interval. Thus, animals began to die in 7 or 8 days, the majority dying in 2 weeks and the remainder in 3 weeks. Several animals survived 4 to 5 or more weeks. In M 520 inoculated with No. 7974, 67 of 117 animals which "took" died within 2 weeks, 27 died in 3 weeks, and

TABLE 4.—*Survival of rats with intraperitoneal transplants of the ascites hepatoma 7974 strain*

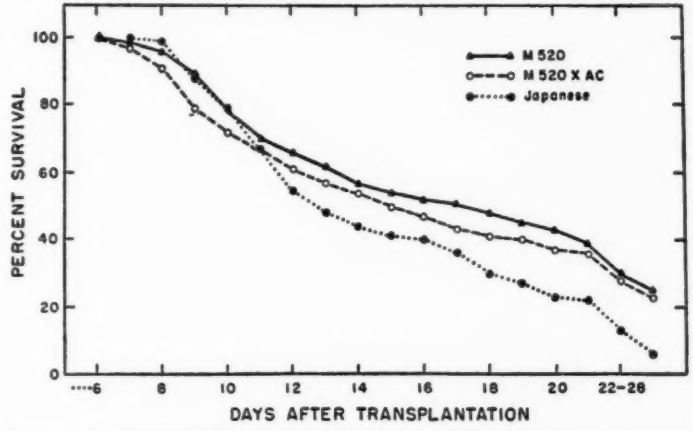
Strain of rats	Number of rats dead with tumor																		Total number of rats
	Days after transplantation																		
	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22–28	29	
Japanese	....	....	1	9	8	10	11	6	3	2	1	4	5	3	2	2	8	6	81
M 520	....	1	5	11	16	13	7	6	8	5	3	1	4	6	3	5	14	9	117
M 520 × AC	....	4	8	15	9	6	8	5	3	5	4	5	3	1	4	1	11	6	98
Sprague-Dawley	....	....	2	3	....	1	1	1	....	....	1	1	....	1	....	....	1	0	12
Holtzman	....	1	....	2	1	....	1	....	....	1	....	1	1	....	....	....	1	0	9
Osborne-Mendel	....	3	....	1	2	1	....	1	1	1	....	....	1	....	....	....	1	0	12

TABLE 5.—*Survival of rats with intraperitoneal transplants of the ascites hepatoma 130 strain*

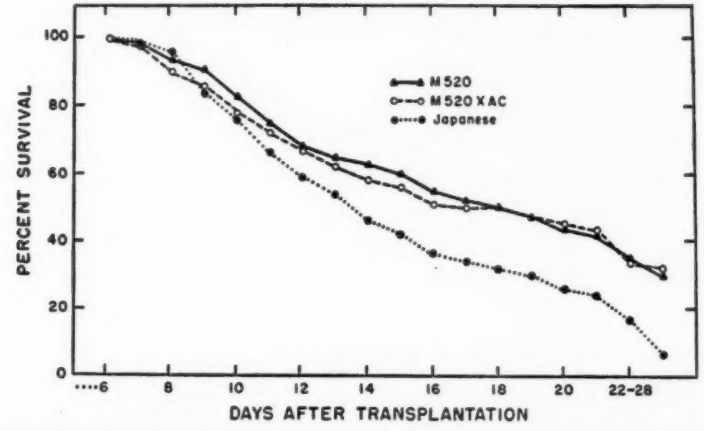
Strain of rats	Number of rats dead with tumor																	Total number of rats
	Days after transplantation																	
	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22–28	29	
Japanese	1	2	9	6	8	5	4	6	3	5	1	2	1	3	2	9	4	71
M 520	2	6	5	11	12	9	5	3	4	7	4	3	4	5	1	11	7	99
M 520 × AC	2	8	4	8	7	5	5	4	2	5	1	0	3	2	2	9	2	69
Sprague-Dawley	....	2	2	1	....	1	1	....	1	1	1	....	1	....	....	0	0	11
Holtzman	....	1	2	1	....	....	....	....	....	....	1	....	....	....	....	0	0	4
Osborne-Mendel	....	1	....	1	....	....	....	....	1	....	1	....	....	....	....	3	2	9

23 in 4 or more weeks. In M 520 × AC strain rats, 58 of 98 animals which "took" died within 2 weeks, 23 within 3 weeks, and 17 in 4 or more weeks. In the case of hepatoma No. 130, similar results were obtained (table 5). These results are similar also to those obtained in Japanese rats. Thus, most of the Japanese rats in which the tumor "took" died about 2 to 3 weeks after inoculation while other rats remained alive for as long as 4 weeks after inoculation, some of these finally dying with tumor.

Rats that died with tumor in the Sprague-Dawley, Holtzman, and Osborne-Mendel strains were few in number, but the values for their survival times were distributed in a manner similar to the strains of rats described above. In text-figures 2 and 3, the percent survival of inocu-



TEXT-FIGURE 2.—Survival curve of rats of different strains intraperitoneally transplanted with the ascites hepatoma (strain 7974).



TEXT-FIGURE 3.—Survival curve of rats of different strains intraperitoneally transplanted with the ascites hepatoma (strain 130).

lated rats of the M 520, M 520 × AC and Japanese strains is indicated. Similar values for the Japanese strain have also been reported by Kaziwara (*13*). In M 520 and M 520 × AC, both curves are similar; whereas in Japanese rats better transplantability is indicated by shorter survival times. With respect to both strains of ascites hepatomas, however, it is clear that their transplantability in the various strains of rats used in this study is essentially the same.

C) *Pathologic findings.*—In the peritoneal cavity of animals that died bearing ascites hepatoma, tumor invasion of the surrounding tissues, often accompanied by a large quantity of tumor ascites, was observed. Typical areas invaded by tumor were the hilus of the liver, as evidenced by a large mass of tumor nodules, as well as in the mesentery and retroperitoneal tissue. Especially characteristic was the appearance of small or large solid nodules on the peritoneal surface and even more frequently on the diaphragm. Tumor invasion to the diaphragm often resulted in an increased pleural fluid which frequently contained hepatoma islands. Distant metastases, *e.g.* to the mediastinum, were also observed. Although differences appeared in the extent and degree to which the ascites hepatoma invaded various tissues, the general picture at autopsy was essentially similar in all strains of rats. In addition, the histologic appearance of tumor tissue in areas of invasion was similar to that of spontaneous hepatomas as previously described in the Japanese rat (*8*, *9*).

Animals that remained alive due to complete, spontaneous tumor regression were sacrificed 2 months after inoculation and examined at autopsy. The majority possessed no ascites in the peritoneal cavity nor was there any indication of tumor invasion.

In a second group of rats which also remained alive for a long period of time (1 to 2 months), the peritoneal cavity contained a large quantity (30 to 50 cc.) of thin ascites free from, or containing very few, tumor islands. In such cases, either several solid tumors were observed or tumor invasion did not occur, but in both instances part of the hilus of the liver and spleen, mesentery, retroperitoneal tissue and the surface of the peritoneum became fibrous and appeared indurated. Microscopically, these portions exhibited increased connective tissue, sometimes accompanied by the presence of a few tumor cells. As in the case of the Yoshida sarcoma, these animals were considered in an intermediate category between those in which true tumor "takes" occurred and those exhibiting complete regression. For practical purposes, however, the intermediate group is considered here to represent essentially a kind of spontaneous regression.

Summary

I. *The Yoshida Ascites Sarcoma*

1) The Yoshida ascites sarcoma was transplanted serially into various American strains of rats for 60 generations.

2) In M 520 and M 520 × AC strains, tumor cells proliferated progressively and the percentage of "takes" was 86 and 82, respectively.

3) In other strains of rats, initial proliferation of tumor cells was followed by regression. In Sprague-Dawley and Holtzman strains, about 50 percent "takes" were obtained. In Buffalo, Wistar, NIH black, and Osborne-Mendel strains, this value ranged between 8 to 15 percent, whereas in O'Grady, Fischer, and Albany strains no "take" was observed.

4) Average survival time for animals with successful "takes" was 9 days with a span of 6 to 14 days.

II. *The Ascites Hepatoma*

1) Two strains of ascites hepatoma of the rat (7974 and 130) were inoculated intraperitoneally into the same American strains of rats and their growth behavior was studied.

2) Both strains of ascites hepatoma exhibited similar growth characteristics in each of the strains tested, as well as in Japanese rats. In rats of the M 520, M 520 × AC, Sprague-Dawley, Holtzman, and Osborne-Mendel strains, the ascites hepatomas proliferated well, reaching a state of nearly pure culture of tumor islands in the peritoneal cavity.

3) The percentage of tumor "takes" was as follows: 75 percent in M 520 and M 520 × AC; 25 percent in Sprague-Dawley, and Holtzman; and 12 percent in Osborne-Mendel.

4) More than 80 percent of the rats which "took," died within 3 weeks after inoculation, while several lived for longer periods of time (30 to 50 days).

5) In Albany, Wistar, O'Grady, Buffalo, and Fischer strains, initial proliferation of tumor islands was followed by spontaneous regression and none of these rats died bearing the ascites hepatoma.

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The *In Vitro* Initiation of Pulmonary Adenomas in Mouse Lung Tissue with Nitrogen Mustard. I. The Influences of Concentration of Agent, Duration of Exposure, and Mitotic State of the Tissue at the Time of Exposure [1, 2]

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Several nitrogen and sulfur mustards have been reported to be carcinogenic in mice (*1*, *2*). The tumors produced were chiefly pulmonary adenomas. The chemicals of this group have also been found to be powerful mutagenic agents in yeast (*3*), bacteria (*4*), and *Drosophila* (*5*). In addition they were widely known as mitotic poisons for the cells of both plants and animals (*6*). These biological effects and the chemical activity (*7*, *8*) of nitrogen mustards have given rise to the suggestion that the mutations and possibly the tumors produced therewith might be brought about through a direct action of this substance upon the chromosomes of dividing cells (*8*).

It was determined to find whether the general methods used in the study of induced genetic changes in unicellular organisms (*3*) would be applicable to investigations of the nature of the oncogenic activity of nitrogen mustard. It is shown that pulmonary adenomas may be initiated by a single brief *in vitro* exposure of mouse lung tissue to nitrogen mustard. The influences of concentration of the agent, the duration of exposure, and the presence of mitoses at the time of *in vitro* exposure are herewith reported.

Materials and Methods

The mice used for the *in vitro* exposure experiments were of the BALB/c (C) strain, bred in this laboratory and procured originally from Dr. Peyton Rous of the Rockefeller Institute for Medical Research, New York City. Their characteristics have been reported elsewhere (*9*). The fetal lung tissue used in these experiments was procured and implanted by the general methods outlined by Rous and Smith (*9*). In this procedure the lungs of fetal and immature animals were finely hashed and exposed *in vitro* to various concentrations of methyl bis(β-chloroethyl)amine hydrochloride (Merck) in Ringer's solution. The time of exposure varied from 15 to 60 minutes. The tissue was then rinsed with a large quantity of Ringer's solution and implanted as a suspension in the posterior thigh muscles of

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young adult mice of the same strain. The implanted animals were killed after an interval of from 2 to 6 months and the implants examined microscopically in serial section for the occurrence and frequency of tumors. These determinations were made without the observer's being aware of the source of the material being studied.

The investigation of *in vivo* effects was carried out in SWR (Swiss) mice bred in this laboratory and procured originally from the Rockefeller Institute for Medical Research. Characteristics of this strain have been previously described (*10*).

In Vitro *Initiation of Adenomas and Influence of Concentration of Agent*

In previous studies of the oncogenic influence of nitrogen mustard a parenteral route of injection had been used (*1*, *2*). Because of its great reactivity and instability (*7*) it seemed possible that the action of this agent might be through an intermediary substance or a complex of substances. If the action of nitrogen mustard were direct, it would appear theoretically possible to produce lung tumors by direct *in vitro* exposure of the tissue to appropriate concentrations of the oncogen. The following experiment was designed to test this assumption. This had not proved the case in previous experiments with urethan (*11*) unless the serum from rabbits previously injected with the drug, so as to contain the oncogenic intermediate was used. In earlier work by Smith, adenomas were readily produced in lung hashings by means of implantation with methylcholanthrene (*12*).

Experiment 1.—Sixteen mouse fetuses measuring 16 to 24 mm. crown-rump, that is, approximately 15 to 19 days' gestation, were obtained from three pregnant mice killed by decapitation. The lungs of the fetuses were freed from mediastinal tissues, excised, pooled, finely hashed with knives, and rinsed in Ringer's solution. The hashed lung was divided into two parts, one of which was kept in Ringer's solution. The other was subdivided into five equal parts placed in separate petri dishes. Four cc. of freshly mixed nitrogen mustard in Ringer's solution was added to each of the five dishes. The dilutions tested were 1:50,000, 1:100,000, 1:500,000, 1:1,000,000 and 1:5,000,000. The exposures were carried out at room temperature. After 15 minutes' exposure the hashings were separated from the mustard solutions, washed for 5 minutes in Ringer's solution, and then suspended in 0.8 cc. of fresh solution. The maximum final concentration of mustard theoretically present after washing was calculated to be less than 1 part in ½ billion. From each of these exposed groups 0.2 cc. of the tissue suspension was implanted intramuscularly in one posterior thigh of three mice. The opposite thigh was implanted with an equivalent amount of a suspension of the hashed lung kept in Ringer's solution and not exposed to mustard. Ten weeks later the mice were killed and their implants examined for tumors. The results are shown in text-figure 1. The relative proportion of well-formed alveolar septa is indicated under the designation "Condition of Implant." Three descriptive classes—good, fair, and poor—were used.

Typical pulmonary adenomas were found in some of the grafts exposed *in vitro* to mustard (text-fig. 1). Implants of material exposed to highest concentrations of the agent were relatively more fibrous and contained fewer alveolar cells. No adenomas appeared in the implants of lung tissue from the same animals not exposed to mustard. In many concurrent experiments of this sort, with other aims, in mice of the same stock no spontaneous adenomas have been found in less than 10 months following implantation of fetal lung (*11*).

The maximum concentration of nitrogen mustard tolerated was found to be 1:500,000. The lethal effects of this and higher concentrations, as manifest by the inhibition of growth or by death and absorption of the implants, indicated that *in vitro* penetration of the tissue by the agent was of a high order if not complete. No effects attributable to differences in concentration within this range were manifest in the number of tumors which occurred in the small groups of animals studied. The smallness of this number of implant hosts belies the fact, however, that thousands of cells from the same animals, but treated *in vitro* in different ways, make up the compared samples.

Experiment 2.—The lungs of nine 1-day-old mice were hashed separately and each specimen divided into two parts. One of these was exposed to 1 part nitrogen mustard in 2½ million parts Ringer's solution and the other to 1 in 10 million. The time of exposure was 30 minutes. After rinsing, the hashings were implanted in opposite thighs of the same mouse. No differences were noted in the size or condition of the implants of the two groups.

The mice were killed 8 weeks after implantation. Only one tumor appeared, this being in an implant of lung exposed to the higher dilution. This was consistent with the lack of critical effect of concentration of the agent upon the relative responsiveness found in experiment 1.

Influence of Duration of Exposure

The lack of a greater influence of concentration of the agent upon the responsiveness suggests that enough mustard was present in the range tested in experiments 1 and 2 to have initiated neoplastic change in many more cells than actually proved susceptible to its oncogenic influence. The question arises as to why so few adenomas occurred in these implants. It is clear that immense numbers of alveolar cells underwent no neoplastic change, yet these cells are of the sort from which these tumors arise (*13*, *14*). The fact that so few cells became neoplastic despite good penetration by the agent suggests that the physiologic condition of the individual cells at the time of exposure to nitrogen mustard may be a critical factor in their reaction. Should a specific, though transient, state be necessary for the oncogenic action of mustard, it would be expected that the number of tumors produced might be increased by lengthening the duration of exposure.

The effect of increasing the duration of exposure was put to test in the following two experiments.

CONCENTRATION NITROGEN MUSTARD**

1 : 5 X 10^6

MOUSE	IN VITRO EXPOSURE	IMPLANT Size Cond.	Adenomas No.	Adenomas size mm.
1	Mustard	Good	1	0.6
	Ringers	Good	none	–
2	Mustard	Good	1	1.0
	Ringers	Good	none	–
3	Mustard	Good	none	–
	Ringers	Good	none	–

1 : 1 X 10^6

MOUSE	IN VITRO EXPOSURE	IMPLANT Size Cond.	Adenomas No.	Adenomas size mm.
4	Mustard	Good	2	0.30 0.25
	Ringers	Good	none	–
5	Mustard	Good	none	–
	Ringers	Good	none	–
6	Mustard	Fair	none	–
	Ringers	Fair	none	–

1 : 5 X 10^5

MOUSE	IN VITRO EXPOSURE	IMPLANT Size Cond.	Adenomas No.	Adenomas size mm.
7	Mustard	Fair	1	0.3
	Ringers	Good	none	–
8	Mustard	Trace Poor	none	–
	Ringers	Poor	none	–
9*	Mustard	Poor	none	–
	Ringers	Fair	none	–

CONCENTRATION MUSTARD 1 : 1 X 10^5

MOUSE	IN VITRO EXPOSURE	IMPLANT Size Cond.	Adenomas No.	Adenomas size
10	Mustard	none present		
	Ringers	Good	none	–
11	Mustard	none present		
	Ringers	Good	none	–
12	Mustard	none present		
	Ringers	Good	none	–

1 : 5 X 10^4

MOUSE	IN VITRO EXPOSURE	IMPLANT Size Cond.	Adenomas No.	Adenomas size
13	Mustard	none present		
	Ringers	Good	none	–
14	Mustard	none present		
	Ringers	Good	none	–
15	Mustard	none present		
	Ringers	Good	none	–

** METHYL BIS(β-CHLOROETHYL) AMINE. HYDROCHLORIDE IN RINGERS

* MOUSE DIED 1 MONTH AFTER IMPLANTATION

TEXT-FIGURE 1.—The direct *in vitro* action of various concentrations of nitrogen mustard to initiate pulmonary adenomas in mouse lung tissue.

Experiment 3.—Sixteen fetuses 16 to 22 mm. crown-rump were procured from two pregnant females. Their lungs were excised, pooled, and hashed in Ringer's solution as before. The hashings were divided into four equal lots, three of which (lots 1, 2, and 4) were exposed simultaneously in 15 cc. centrifuge tubes to 5 cc. 1 part nitrogen mustard in 5 million Ringer's solution. Mustard was added to lot 3 thirty minutes later. The fragments were agitated every 5 minutes. The duration of exposure to the mustard solution was 15 minutes for lot 1, 30 minutes for lots 2 and 3, and 1 hour for lot 4. The exposure of each lot was terminated by centrifuging at 1,000 r.p.m. for 2 minutes, after which the hashings were drained, and 10 cc. Ringer's added to the 0.2 cc. drained hashings in the tube. The tissue was then rinsed with agitation for 5 minutes, centrifuged again, drained, and the hashings suspended in 1 cc. of Ringer's solution. Lot 2 was similarly rinsed and suspended 30 minutes after addition of mustard. Lot 3, that to which the mustard was added 30 minutes after lots 1 and 2, was similarly rinsed at the same time as lot 4, which received an hour's *in vitro* exposure to the mustard. The suspensions of hashed lung in Ringer's solution from lots 1 and 2 were implanted opposite one another in the thigh muscles of five adult mice. Lots 3 and 4 were also implanted opposite one another in the legs of four other animals. Seven weeks later the mice were killed and the implants examined for adenomas. The results are shown in table 1.

TABLE 1.—*Duration of* in vitro *exposure to nitrogen mustard* and number of tumors initiated*

Host of implant	Lot 1		Lot 2	
	Exposure time (*minutes*)	Adenomas (*number*)	Exposure time (*minutes*)	Adenomas (*number*)
1	15	0	30	1
2	15	0	30	1
3	15	1	30	0
4	15	0	30	1
5	15	0	30	0
	Lot 3		Lot 4	
6	30	3	60	1
7	30	2	60	0
8	30	0	60	1
9	30	0	60	0

* Nitrogen mustard concentration: 1 part in 5 million parts Ringer's solution.

Experiment 4.—Seventeen mouse fetuses measuring 20 to 22 mm. crown-rump of approximately 18 to 19 days' gestation and procured from two pregnant BALB/c mice were used. The lungs of the fetuses were excised, hashed and after division into four equal parts transferred to fresh petri dishes. Three cc. of a solution containing 1 part nitrogen

mustard in 2½ million parts of Ringer's solution was added at once to two lots, A and D, to be exposed for 15 minutes and 60 minutes, respectively. Fifteen minutes later, one of these (A) was washed in 10 cc. of Ringer's solution as in experiment 3, suspended in 1.5 cc., and kept in the petri dish. A fresh batch of the original solution of mustard was then added to a third lot (C), which was exposed for 30 minutes. Forty-five minutes after the addition of mustard to the first two lots, it was added to the fourth lot (B), which received a 15-minute exposure to mustard terminated at the same time as that exposed for 60 minutes. All were washed, suspended in 1.5 cc. of Ringer's, and implanted in 0.2-cc. amounts in the thigh muscles of fifteen young adult mice. As in the previous experiment, materials exposed to mustard for differing periods were implanted opposite one another. Six months later the mice were killed and the implants examined for tumors. Again pulmonary adenoma was the only type of tumor found. The results are shown in table 2 in which, because of the complexity of the experimental design, the results are shown pooled rather than in relation to the hosts carrying the implants.

TABLE 2.—*The influence of duration of* in vitro *exposure to the oncogen upon the number of tumors initiated*

Lot	A	B	C	D
Time exposed* (*min.*)	15	15	30	60
Adenomas	1	0	3	5

* Nitrogen mustard concentration: 1 part in 2.5 million Ringer's solution.

Judging from the above experiments and those to follow, the implant host exerted no perceptible influence upon the response of the *in vitro* exposed implanted lung tissue.

The results of experiments 4 and 5 make clear that increasing the duration of *in vitro* exposure increases the number of tumors initiated. Though the number of tumors produced in these experiments is too small for detailed quantitative evaluation, it seems noteworthy that the number of tumors, though increased by allowing a 60-minute exposure is not increased as much as one might have anticipated. Indeed in experiment 3 (table 1) somewhat fewer tumors occurred in the hashings exposed for 60 minutes than in those exposed for 30 minutes. Though some inactivation of the agent during the time of exposure no doubt occurred, the absence of any critical effects of diminishing its concentration in experiments 1 and 2 makes it unlikely that inactivation accounts for these results.

It seemed that a possible explanation for these findings might lie in the observation of Bullough who found that the number of new mitoses rapidly diminishes in tissues kept *in vitro* in a balanced salt solution (*15*). The question arises whether the initiation of these tumors with mustard might be related to cell division. Experiments were undertaken to ascertain whether or not a reduction in the mitotic rate would have an effect on the *in vitro* responsiveness of pulmonary tissues to nitrogen mustard.

Influence of Mitotic Rate

The number of mitoses can be profoundly reduced by fasting mice for 12 to 36 hours (*16*). In the following experiments this influence of fasting was used to reduce the mitotic rate of pulmonary cells.

Experiment 5.—Ten 3- to 4-day-old mice were separated from their mothers and fasted for 24 hours. Ten mice of the same size and age and from the same litters were allowed to feed *ad libitum* up to the time of excision of their lungs. The lungs of each group were excised, hashed, rinsed with Ringer's solution and exposed for 30 minutes to 1:2,500,000 mustard-Ringer's. The fragments were then rinsed in equal volumes of Ringer's and suspended in 2 cc. of this solution. The hashings from the fasted animals were implanted opposite those from the full-fed mice in the thigh muscles of 10 young adults. Blocks taken from the unexposed hashings of each group at the time of addition of mustard revealed many mitotic figures in the tissue from full-fed mice but none were seen in the tissue from the fasted animals; twenty high-power fields were examined in each sample. Five months later the 10 implanted mice were killed and the implants examined for adenomas. The implants from group to group were of the same size and condition.

The implants of lung tissue from full-fed animals yielded seven adenomas in four positive implants. No tumors were found in the implanted tissue from the fasted group. This finding concerning the effect of a reduced mitotic rate, as produced by fasting, upon the initiation of the neoplastic change was confirmed by experiment 6.

Experiment 6.—Eight litters of mice 1 week old were equally distributed in two groups of 20 mice each. Those in one group were left to suckle their mothers. Those in the other group were fasted as in experiment 5. After 24 hours the lungs of each group were excised and exposed to 1:500,000 nitrogen mustard for 20 minutes. As the lungs were removed, blocks were taken for mitotic counts. After rinsing and suspension in fresh Ringer's solution the materials were implanted opposite one another in the thighs of 20 young adult mice. Mitotic counts in these tissues revealed the profound effects of fasting on the mitotic rate. Between 1 and 2 mitotic figures were seen per high-power field in the lungs from full-fed mice, while only rare ones were found in the lungs of the fasted animals. Seventeen mice remained when the experiment was terminated 2 months after exposure of the lung to nitrogen mustard. The aggregate amount of surviving tissue of each group was essentially the same. All implants were in good condition.[3]

In table 3 it is again evident that the responsiveness of the lungs with relatively high mitotic rates at the time of *in vitro* exposure greatly exceeded that of the tissue in which the mitotic rate had been diminished by fasting.

[3] Their state contrasted with the relatively poor condition of the implants exposed to this concentration of mustard in experiment 1. The variability of effect of mustard concentration when used on different occasions has been previously reported (*5*).

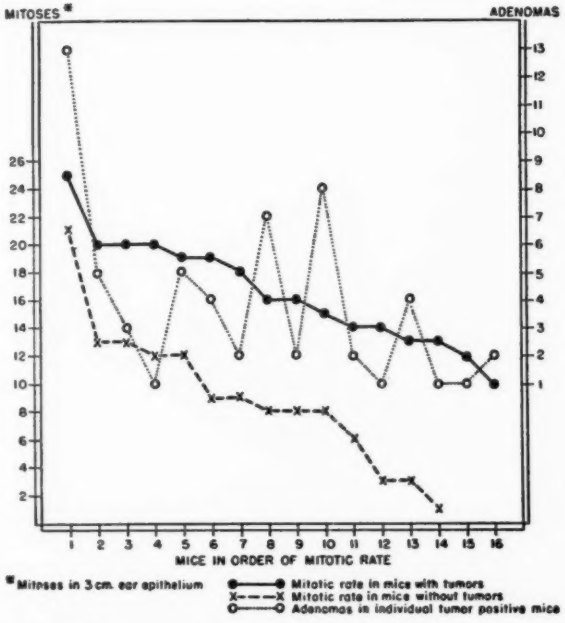
TABLE 3.—*The influence of the mitotic rate of the exposed tissue upon the number of tumors initiated, fasting being used to reduce the mitotic rate of the pulmonary cells*

Implant host	Lung of fasted mice	Lung of full-fed mice
	Adenomas	
1	0	1
2	0	2
3	0	3
4	0	0
5	1	0
6	0	1
7	0	2
8	0	0
9	0	0
10	0	2
11	0	1
12	0	0
13	1	1
14	0	0
15	0	0
16	0	0
17	0	No implant found

Fasting, however, has many influences other than to reduce the incidence of mitoses. Because of this fact it was determined to find whether the number of adenomas produced by the exposure of full-fed mice *in vitro* to the oncogen would be correlated with their growth rates at the time of exposure. It had been previously shown elsewhere in relation to its nuclear poisoning effects that nitrogen mustard is effectively out of the blood stream within 15 minutes of the time of injection (*17*).

Experiment 7.—Forty Swiss males 4 to 5 weeks old were given 0.004 mg. of nitrogen mustard per gram of body weight of mouse intravenously in the tail vein. Immediately before injection of the agent a block of tissue was taken from the right ear of each mouse for microscopic examination and mitotic counts. A cross section of skin 3 cm. in total length was examined from each animal, half of this being from the inner and half from the outer surface of the ear. This length was achieved by the examination of several histologic sections taken from the same biopsy. Only mitoses in epithelial cells were recorded. It had been previously shown elsewhere that the mitotic rate of the epithelium could be used as a rough index of the relative mitotic activity of the various organs of the same individual for any given time (*16*). Thirty animals remained when the mice were killed for examination 9 weeks after injection. Nine had been discarded because of intercurrent disease or dubious intravenous injection at the start of the experiment. One was discarded because of microscopic evidence of previous recent injury to the ear before the time of biopsy. The lungs were examined grossly for the presence of tumors on the pleural surface. Only those one-third mm. in diameter (and over) figure in the results, as smaller tumors may be confused with lymphoid aggregates.

Sixteen mice had tumors and 14 had none. The number of tumors ranged from none to 13. The tumor-positive group averaged four adenomas per mouse. The average number of mitotic figures in the 3 cm. of ear epithelium examined was 17 in the case of the animals developing tumors and 9 for the tumor-negative group. The results for the individual mice are shown in text-figure 2. It is clear that the animals developing tumors were in general those with the higher mitotic rates at the time of exposure to the oncogen.



TEXT-FIGURE 2.—The relation between the mitotic activity of mice at the time of *in vitro* exposure to nitrogen mustard and the response of tumor-positive and tumor-negative animals.

On comparison of individual tumor-positive animals in relation to their mitotic activity at the time of injection and the number of adenomas produced, a rough general correlation may be seen. The correlation coefficient for this relation is 0.85. This is beyond the 0.1 percent point and statistically highly significant. On comparing these positive mice groupwise the relation becomes again apparent. The 8 positive mice with the higher mitotic rates developed 38 adenomas while the 8 animals with the lower rates developed only 21. The average mitotic rates of these two groups was 20 and 13, respectively. There is reason to believe that were it not for the nonspecific reactions occurring between the nitrogen mustard and blood and tissue components (*8, 17*) the correlation among the tumor-positive animals would have been much more precise.

Discussion

These experiments establish the fact that the general methods used in the study of induced genetic changes in unicellular organisms may be applied to the study of oncogenesis. A brief *in vitro* exposure of fetal and neonatal pulmonary tissue to high dilutions of nitrogen mustard suffices to initiate neoplastic change. The influences of duration of exposure (experiments 3 and 4) and of the presence of mitoses in the exposed tissue (experiments 5 and 6) make plain that the oncogenic influence of the agent was exerted only during the brief period of exposure and not over an indeterminate period of time because of adsorption of the drug on the tissues. The delineated lethal effects of increasing concentrations of the oncogen (experiment 1) show that penetration by the agent was good.

Although there was universal exposure of alveolar septal cells to an effective concentration of nitrogen mustard, the number of alveolar septal cells undergoing neoplastic change was remarkably small. Inasmuch as the incidence of tumors increased with increases in the duration of exposure and in the range tested and was not critically dependent on the concentration, it appears likely that the determining factor is a function of a specific but transient physiologic cell state. The correlation between cell division at the time of exposure and the number of tumors initiated by the oncogen seems particularly pertinent (experiments 5, 6, and 7). The lack of responsiveness of lung tissue of the animals less than 1 day old (experiment 2) is consistent with the result, as it is known that newborn mice have relatively low mitotic rates (*18*). Though these experiments do not necessarily mean that the nitrogen mustard acted directly upon the chromosomes of cells during division, it does appear that some part of the cell becomes more vulnerable to its oncogenic influence near the time of cell division. Whether this period of increased vulnerability is shortly before, during, or shortly after the time of actual division cannot be inferred.

The relation between the oncogenic action of nitrogen mustard and cell division appears clearer when it is considered that morphologically indistinguishable pulmonary adenomas may be initiated by a single brief *in vitro* exposure of fetal lung tissue to 2,537 Å ultraviolet irradiation (a wavelength in the range of maximum absorbance by nucleic acid) (*19*). It is also pertinent that the initiation of tumors of this same sort with urethan is profoundly influenced by precursors to the nucleic acids, some nucleic acid components, and by certain substances affecting their synthesis (*20*, *21*).

Summary

Certain of the general methods used in studies of induced genetic changes in unicellular organisms are shown to be of value in investigations concerning the nature of the oncogenic influence exerted by nitrogen mustard on the lung tissue of mice.

A single *in vitro* exposure of mouse lung tissue to nitrogen mustard, methyl bis(β-chloroethyl)amine, for as little as 15 minutes, suffices to

initiate pulmonary adenomas. Within the range of dilution tested the production of tumors is not critically dependent on the concentration. An increased time of exposure to the agent results in an increased number of tumors. Since penetration of the nitrogen mustard was good (as judged from its lethal effects), the relatively small number of cells undergoing neoplastic change seems to indicate that a specific cell state at the time of exposure to the drug is an essential factor in the occurrence of the neoplastic change. Experiments indicate that the oncogenic action of nitrogen mustard is in some way conditioned by the occurrence of cell division during the time of exposure to the agent.

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Neoplasms in Mice Inoculated with Cell-Free Extracts or Filtrates of Leukemic Mouse Tissues. I. Neoplasms of the Parotid and Adrenal Glands[1]

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In 1951, Gross (*1*) described spontaneous leukemias in C3H mice following the inoculation in infancy with extracts prepared from leukemic or embryonic tissues from strain AK-n mice. That same year the author attempted to confirm Gross's findings. Of 42 newborn C3H mice inoculated when less than 8 hours old, 14 lived for 10 months or longer, a few being observed for as long as 30 months. None of these 14 developed leukemia, nor did any of those that died before the age of 10 months develop leukemia. However, 5 of the 14 mice developed tumors of the parotid gland of a type not previously described in mice, and 1 of the mice had bilateral tumors of the adrenal glands. The parotid-gland tumors were not reported at that time, awaiting repetition of the experiments. These observations were made independently of Gross's recent description (*2*) of salivary gland tumors in C3H mice following inoculation with AK-n leukemic extracts or filtrates; in his earlier work Gross described leukemias only (*1, 3–5*).

Tumors of the salivary glands designated as myo-epitheliomas are known to occur spontaneously in mice of strains A and C but have not been reported as occurring in strain C3H or strain AKR. The neoplasm of the parotid gland arising in mice of strains C3H and C3Hf, which had been inoculated with extracts or filtrates of leukemic tissues from strain AKR or C3H mice, are strikingly pleomorphic and unlike any tumor previously seen in untreated mice, although some of the tumors have areas which resemble myo-epitheliomas in the cellular arrangement.

In the work reported here, some of which was previously reported in abstract form (*6*), the following observations have been made: 1) An incidence of 2.6 percent of leukemia was produced in strain C3H mice following the technique described by Gross (*1, 3–5*). This is close to the normal incidence of leukemia in this strain of mouse, but the leukemia appeared in our mice at 3 months of age, which is unusually early for spontaneous leukemia. The leukemias were specific for strain C3H mice;

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i.e., when transplanted into adult mice (2 to 4 months old) of strains AKR and C3H, they grew only in the C3H mice. 2) Neoplasms of the parotid gland were observed in 21 of 79 strain C3H or strain C3Hf mice that had reached maturity and had been inoculated shortly after birth with extracts or filtrates prepared either from AKR leukemic tissues or from C3H leukemias. 3) In six experiments, in which one or more litters of newborn C3H mice (less than 8 hours old) were inoculated with extracts or filtrates from a transplanted C3H leukemia, an incidence of 36 percent of adrenal-gland tumors and 36 percent of parotid-gland tumors was encountered. Many of the mice had both parotid- and adrenal-gland neoplasms, in some instances bilateral in both organs. 4) Tissue extracts from apparently normal C3H mice inoculated into newborn strain C3H mice have, in no instance, produced tumors of either the parotid gland or the adrenal gland.

The adrenal-gland neoplasms observed in mice of strains C3H and C3Hf inoculated soon after birth with extracts or filtrates prepared from a transplanted C3H leukemia appear to be of a type not described previously. Early tumors appeared to be confined to the medulla. The tumor cells gave a negative chromaffin reaction; so, if of medullary origin, they are not of the mature cell type as are the pheochromocytoma. Some of these neoplasms metastasize widely, as will be shown later in the illustrations.

In strain C3H mice, spontaneous tumors of the adrenal glands—either cortical or medullary in origin—are relatively rare in untreated animals. In over 100 control mice of strains C3H and C3Hf autopsied in this laboratory (histologic sections having been examined from the different organs, including the salivary glands and adrenal glands), no adrenal tumors were encountered. In a review of the literature, Smith, Gardner, Li, and Kaplan (*7*) found a remarkably low incidence of adrenal-gland tumors of medullary origin even in experimental animals. They were able to induce a small percentage of medullary tumors in mice of the BRS stock after castrating, irradiating, and treating the mice with hormones. Woolley *et al.* (*8–12*) have found great variation in adrenal-cortical growth and differentiation following early gonadectomy in certain inbred strains of mice. In some strains there was only nodular hyperplasia of the adrenal cortex and, in others, adrenal-cortical carcinoma. Changes in the adrenal medulla were not described.

Materials

Mice.—With the exception of a few strain AK-n mice bearing spontaneous leukemias that were obtained from Dr. Ludwik Gross, Veterans Administration Hospital, Bronx, New York, all mice were obtained from stocks at the National Institutes of Health—strain C3Hf and strain AKR from Dr. L. W. Law, and strain C3H from Dr. G. E. Jay. The substrains of C3H and C3Hf, used as breeders for the newborn, were established from mice obtained from these sources. The animals were housed in plastic cages at a temperature of 78° F. They were supplied with

Purina laboratory chow and tap water *ad libitum* and were given greens twice weekly. The newborn mice used for inoculation were generally less than 8 hours old; none were over 12 hours old except in a few limited experiments, and in these instances the ages are given. In all experiments, except where otherwise stated, the inoculations were subcutaneous, the needle being introduced into the back just above the base of the tail and the material deposited at the back of the neck. The skin punctures were sealed with collodion.

Leukemic extract and filtrates.—The extracts from strain AKR (or AK-n) mice were prepared from mice bearing either spontaneous or transplanted leukemias. The extracts from the C3H leukemias were from transplanted leukemias only.

Methods and Results

Attempted Confirmation of Gross's Observation on Leukemia

In each instance the enlarged liver, spleen, thymus, mesenteric nodes, peripheral nodes, and the transplanted tumor, if present, were combined and homogenized in a Potter-Elvehjem homogenizer and enough physiological saline or Ringer's lactate solution (Lilly) was added to make a 20-percent suspension. In the different experiments the homogenized suspension was further treated by various procedures that will be described in each case. Aseptic techniques were observed throughout. Twenty-percent homogenized suspensions were prepared from AKR leukemic tissues and centrifuged in a PR-1 International Centrifuge at 3,000 r.p.m. (1,400 × g) at 0° C. for 15 minutes. In the early experiments extracts were prepared by removing such supernatants and recentrifuging at 3,000 r.p.m. for 15 minutes at 0° C. This second supernatant containing the extract was used for inoculating the newborn mice. Such extracts are designated as extract (*a*), table 1. In later experiments the second centrifugation was carried out at 9,500 r.p.m. (7,000 × g) for 10 minutes at 0° C. Such supernatants are referred to as extract (*b*), table 1. In still later experiments, the supernatants from the second centrifugation at 9,500 r.p.m. were filtered through Selas filters of #03 porosity and used as filtrates for inoculating newborn mice. A total of 128 newborn C3H and C3Hf mice, from 3 to 12 hours old, were inoculated subcutaneously with 0.1 ml. of extract or filtrate. Of these, 98 received extracts and 30 received filtrates. Those surviving the inoculum have been under observation from 12 to 30 months. Two of the mice inoculated with extract developed a generalized leukemia when 3 months old. These leukemias are specific for C3H mice; that is, on transplant they will grow only in C3H mice. A summary of these experiments is given in table 1.

Leukemia in Mice Produced by Inoculated Fetal Tissue Transplanted to the Anterior Chamber of the Eye

To increase the possibility of demonstrating a leukemia-producing agent in AKR leukemic tissues, an extract was prepared from 6 pooled

AKR leukemias—1 transplanted leukemia, #L4616 (obtained from Dr. L. W. Law, National Cancer Institute, and in its 8th transplant generation) and 5 spontaneous leukemias. A 20-percent, homogenized suspension was prepared, as described above, and was centrifuged at 3,000 r.p.m. (1,400 × g) at 0° C. for 15 minutes. The supernatant was transferred to a second tube and recentrifuged at the same speed for the same length of time. Only half the recentrifuged supernatant was used as extract and the rest was filtered through a Morton glass filter of fine porosity and used as filtrate. The sediment was saved for use in another experiment. The above leukemic extract and filtrate were tested for the presence of a leukemia-producing agent by the following method.

TABLE 1.—*Leukemia in strain C3Hf and C3H mice inoculated when from 3 to 12 hours old with extracts or filtrates from AKR leukemic tissues*

Expt. No.	Inoculum	Strain of mouse inoculated	Mice inoculated	Mice surviving	Mice with leukemia	Age at which leukemia developed (*months*)
1	Extract (*a*)	C3H	20	10	0	
2	"	"	6	0	0	
3	"	"	7	7	0	
4	"	"	3	3	0	
5	"	"	11	11	0	
6	Extract (*b*)	"	7	3	1	3
7	"	C3Hf	3	3	0	
8	"	C3H	8	5	0	
9	"	"	5	5	0	
10	"	"	10	8	1	3
11	"	"	18	4	0	
12	Filtrate	C3Hf	15	10	0	
13	"	C3H	10	4	0	
14	"	"	5	5	0	
Total....	.....	.....	128	78	2	

Under aseptic conditions six C3H fetuses, each 20 mm. long, were removed from a normal C3H female. The thymus and spleen were removed from each fetus and placed in sterile tissue dishes. Two sets of tissue dishes were prepared with the leukemic extract and two with the filtrate, one in each set receiving two of the spleens, and the other receiving two of the thymus glands. Two spleens and two thymus glands were put in a third set of dishes, which contained only physiological saline solution. These fetal organs were then cut into pieces small enough to transplant into the anterior chamber of the eye of an adult mouse. It was felt that cutting up the tissues in the extract or filtrate would increase the chances of inoculating the fetal tissues with a leukemic agent. After being cut, the tissues were allowed to stand in the preparation for 15 minutes or longer before transplantation.

The transplants were made into the anterior chamber of the eyes of 10-week-old strain C3H males. Orally controlled pipettes were used, and the tissue was introduced along with a small amount of the fluid in

which it had been immersed; the eye chamber was then flushed with more of the same fluid.[3] A total of 36 mice was inoculated; the results are shown in table 2.

TABLE 2.—*Leukemia in C3H mice inoculated into the anterior chamber of the eye with fetal tissue plus AKR leukemic extract or filtrate*

Fetal tissue	Fluid	Number of mice inoculated	Number surviving 10 months*	Number developing leukemia	Leukemia on further observation
Spleen.....	Extract....	6	5	0	0
Spleen.....	Filtrate....	6	2	0	0
Thymus.....	Extract....	6	4	2†	0
Thymus.....	Filtrate....	6	4	0	0
Spleen.....	Ringer's lactate.	6	3	0	0
Thymus.....	Ringer's lactate.	6	4	0	0

*Autopsies on mice dying before 10 months generally showed pneumonia; none showed leukemia.
†At 10 months.

Of the 6 mice receiving fetal thymus inoculated with leukemic extract. 2 developed a generalized leukemia 10 months after the transplantation, These were leukemias #17 and #19 (corresponding to the mouse numbers). They are lymphatic leukemias which on transplantation grow only in strain C3H mice. The experiment has been repeated with similar results. Of 20 C3H mice inoculated in the anterior chamber of the eye with transplants of C3H fetal thymus that had been immersed in AKR leukemic extract, 2 developed a generalized leukemia at 12 and 14 months, respectively, after inoculation. These were also lymphatic leukemias which on transplantation grew only in strain C3H mice. Twenty control mice that received fragments of the same thymi immersed in saline and 20 that received only AKR leukemic extract in the anterior chamber of the eye have remained free of leukemia in a 21-month observation period. Whether these leukemias arose from a leukemia-producing agent in AKR tissues cannot be ascertained at this time. The primary site of origin of the leukemias did not appear to be in the anterior chamber of the eye, but they appeared as generalized leukemias.

The origin of these leukemias is given in detail because extracts and filtrates from one of these, leukemia #19 used in other experiments (described in a later section), have given interesting results. The other leukemias have not been used experimentally. Leukemia #19, at the time of this report, is in its 87th transplant generation in a period of 28 months. Photomicrographs of sections of the organs of a mouse carrying leukemia #19 in its 50th transplant generation are illustrated in figures 1 and 2. Subcutaneous transplants of this leukemia will kill in from 8 to 10 days 100 percent of strain C3H mice 8 to 10 weeks old. In the terminal stages these mice have hemorrhagic local tumors at the site of inoculation, enlarged hemorrhagic peripheral nodes, enlarged spleens,

[3] Transplantation operations were performed by Dr. Clifford Grobstein of this Institute.

and enlarged pale livers. The kidneys also are very pale. Smears of the peripheral blood show a marked increase in the white blood cells, the predominating cells being lymphoblasts.

Parotid-Gland Tumors in C3H Mice and C3Hf Mice

Neoplasms of the parotid glands have been produced in strain C3H mice with the following extracts or filtrates:

1) *Filtrate from pooled AKR leukemias.*—Part of the sediment from the centrifugate of the 6 pooled AKR leukemias used in the preceding experiment was resuspended in saline to make a 20-percent suspension, quickly frozen in CO_2 ice and alcohol, and then quickly thawed at 37° C. in order to break up the cells further and thus release any agent present within the cells. This frozen and thawed extract was stored at 4° C. for 72 hours to allow elution from the cell fragments of any agent present, and was then centrifuged for 15 minutes at 3,000 r.p.m. (1,400 × g) at 0° C. The supernatant, which was clear, was then filtered through a Morton glass filter and the filtrate was bottled and stored at 20° C. for 3 days, awaiting newborn C3H mice for inoculation. The frozen filtrate (which by then had been extracted 6 days previously) was thawed and 0.1 ml. was inoculated intraperitoneally into 22 newborn (less than 8 hours old) C3H mice (table 3). Twelve mice survived the inoculation. Twelve months later, 9 remained alive, 3 having died of pneumonia or mammary tumors. At 12 months, 3 male mice of this group were found to have tumor masses in the parotid region, one of them having bilateral tumors. The 6 remaining were observed up to 30 months and no leukemias, tumors of the parotid gland, or other unusual tumors developed in these mice.

2) *Extract from a transplanted AKR leukemia.*—A 20-percent homogenate was prepared from transplanted AKR leukemia #L4616 (Dr. L. W. Law, National Cancer Institute) which was in its 11th transplant generation. The homogenized suspension was centrifuged at 3,000 r.p.m. (1,400 × g) for 15 minutes at 0° C., after which the supernatant was removed and recentrifuged for 15 minutes at the same speed. Twenty newborn strain C3H mice, less than 8 hours old, were inoculated intraperitoneally with 0.1 ml. of this extract, and 10 survived the inoculation. Nine months later only 5 mice remained alive, 5 having died of pneumonia or mammary tumors. Among these 5 surviving mice, 2 males developed parotid tumors (1 bilaterally) when 10 and 12 months old, respectively. One female, 11 months old, was found to have a large mammary tumor in the perineal region, bilateral tumors in the adrenal glands, bilateral tumors in the ovaries, and enlarged peripheral nodes (table 3). Transplantations and histologic studies made from the adrenal tumors, the ovarian tumors, and the lymph nodes showed these tumors to be of the same cell type. These are morphologically the same as the adrenal tumors found in later experiments.

3) *Extract and filtrate from AK-n leukemia #60 (Gross).*—An AK-n mouse bearing a spontaneous leukemia was obtained from Dr. L. Gross. Both extracts and filtrates were prepared from this leukemia. A 20-percent

homogenate was prepared from the leukemic tissues. It was then centrifuged at 3,000 r.p.m. (1,400 × g) for 15 minutes at 0° C., the supernatant fluid was removed and centrifuged at 9,500 r.p.m. (7,000 × g) for 10 minutes at 0°C., and then filtered through a Selas filter (#03 porosity); 0.1 ml. of the filtrate was inoculated subcutaneously into 15 newborn C3Hf mice. Of the 10 mice surviving, which are now 14 months old, none has developed tumors.

The sediment from the centrifugate was resuspended in saline to make a 20-percent suspension, quickly frozen in dry ice and alcohol, and quickly thawed at 37° C. It was stored at 4° C. for 7 days, then centrifuged at 3,000 r.p.m. (1,400 × g) for 15 minutes at 0° C., and the supernatant fluid removed and centrifuged at 9,500 r.p.m. (7,000 × g) for 10 minutes; 0.1 ml. of the new supernatant was inoculated subcutaneously into 12 C3Hf mice (table 3). Eight survived to reach maturity and one developed a parotid-gland tumor at 9 months of age; the remaining mice are now 14 months old.

4) *Extract from an AKR leukemia adapted to C3H mice.*—Tumor RIL6, a C3H-adapted tumor (Stewart, *13*), representing the six pooled leukemias described above, was extracted when in its 29th transplant generation and centrifuged as described in section 2; 0.1 ml. was inoculated intraperitoneally into 21 newborn C3H mice no older than 8 hours. Nine of the mice survived; of these, 4 developed parotid-gland tumors, at 5, 8, 10 and 12 months of age, respectively. More in this group may develop parotid tumors, since the experiment is only in its 13th month.

5) *Extracts and filtrates from strain C3H leukemias.*—Since only an occasional leukemia was observed as a result of inoculation with extracts or filtrates from AKR mouse leukemia, it was felt that the incidence of leukemia might be increased if the extracts and filtrates were prepared from leukemias specific for C3H mice.

a) Filtrates from C3H leukemia #1124.—Leukemia #1124 arose as a generalized leukemia in a 3-month-old C3H mouse that had been inoculated intraperitoneally with tumor RIL6 3 weeks previously in the process of adapting the tumor (Stewart, *13*). When transplanted this leukemia will grow only in C3H mice; whereas the adapted RIL6 leukemia after 30 months of transplanting in C3H mice in which it grows progressively, killing in 14 to 16 days, will also grow progressively in AKR mice, killing in the same time. Whether leukemia #1124 arose from the inoculum or as a spontaneous leukemia cannot be ascertained. At the time the extracts were made from leukemia #1124, it was in its 22nd transplant generation. A 20-percent, homogenized suspension was centrifuged at 3,000 r.p.m. (1,400 × g) for 15 minutes at 0° C. It was then filtered through a collodion membrane, prepared by the method of Elford and Ferry (*14*) and having a pore diameter of 350 mμ. Filtration was carried out with a positive nitrogen pressure of between 40 and 45 pounds. Filtration was slow; only 1 ml. passed in 2 hours. Eleven newborn mice, 8 hours old, were inoculated intraperitoneally with 0.1 ml. of the filtrate (table 3). One female mouse developed a unilateral parotid-

TABLE 3.—*Parotid- and adrenal-gland tumors in strain C3H and C3Hf mice inoculated when newborn with extracts or filtrates of mouse leukemia*

Leukemia used for preparing extract	Inoculum	Strain of mouse	Mice inoculated (*number*)	Mice reaching maturity (*number*)	Mice with parotid tumors (*number*)	Mice with adrenal tumors (*number*)	Age at which tumor occurred (*months*)	Living without tumors (*number*)
6 Pooled AKR, including #L4616.	Filtrate from frozen-thawed tissues.	C3H	22	12	3	0	12	0
Transplanted AKR #L4616.	Extract	C3H	20	10	2	1	10, 12, 11	0
AK-n #60 (Gross)	Extract from frozen-thawed tissues.	C3Hf	12	8	1	0	9	6
AK-n #60 (Gross)	Filtrate	C3Hf	15	10	0	0		10
RIL6 adapted to C3H	Extract	C3Hf	14	9	4	0	5, 8, 10, 12	4
C3H #1124	Filtrate	C3H	11	4	1	0	11	0
C3H #19	Filtrates and extract	C3Hf and C3H	70*	25	9	9	9 to 12	3
Total			164†	79	21	10		23

*125 were inoculated but all the mice in 9 litters, representing 55 in number, died within a period of 3 weeks so are not included in the total.
†Of 78 mice surviving (table 1) that were inoculated by Gross's method, none developed parotid-gland tumors.

gland tumor at 11 months of age; none developed leukemia; 3 still survive and are now in their 19th month of life.

b) Extracts and filtrates from C3H leukemia #19.—Extracts and filtrates from this leukemia, which were described above, also produced parotid-gland tumors. These experiments will be described in the section on adrenal-gland tumors, since not only was a high incidence of parotid tumors observed, but also many of the mice inoculated with preparations from this leukemia developed tumors of the adrenal glands. For comparative purposes, description of the parotid tumors will also be given later, with the description of the adrenal tumors. Table 3 summarizes the experiments in which parotid-gland tumors were observed.

Adrenal-Gland Neoplasms in Strain C3H Mice

A total of 125 newborn mice were inoculated with extracts or filtrates prepared from C3H leukemia #19. The mortality was very high. In many instances the whole litter of inoculated infant mice died within 9 to 21 days. Few lesions that could be attributed to the inoculums were found in these mice that died young, although atrophy of the thymus and nucleated red blood cells in the peripheral blood, accompanied by a granulocytosis, were consistent findings. Experiments in which the whole litter of inoculated mice died within a short time were not included in determining the incidence of parotid- or adrenal-gland tumors observed in mice receiving extracts or filtrates from this leukemia. Only mice that lived 3 months or longer are included.

In a series of six experiments covering a period of 6 months, during which leukemia #19 passed from its 7th transplant generation to its 33rd, extracts and filtrates prepared from the different transplant generations were used for inoculating the infant mice. In six of these experiments, where mice 4 to 8 hours old were inoculated, and 25 reached maturity, an incidence of 36 percent of parotid-gland tumors, plus an incidence of 36 percent of adrenal-gland tumors, was observed. Serial sections of the adrenal glands were not done, and some of the adrenal tumors may have been missed. The remaining mice are now 12 to 18 months of age. No leukemias developed in any of the mice receiving the inoculum. The methods for preparing the extracts or filtrates were the same as those already described. Table 4 summarizes the six experiments and gives the method of centrifugation and filtration.

Characteristics of the Parotid- and Adrenal-Gland Neoplasms

Parotid-gland tumors.—Grossly, the tumors are yellowish-white, glistening, separate nodules or clusters, which may be quite firm and solid on cutting or may be filled with a yellow-green fluid or a slimy mucinous substance. Figures 3 to 10 show the gross and microscopic appearance. The spontaneous tumors grow very slowly; a mouse may survive 4 to 5 months from the time the tumor is first noted. If mice bearing these tumors—either spontaneous or transplanted—are allowed to live until

TABLE 4.—*Tumors of the parotid and adrenal glands produced in strain C3H and C3Hf mice inoculated when newborn with filtrates and extracts of C3H leukemia #19*

Transplant generation of leukemia	Treatment of leukemic homogenates	Strain of mice	Mice inoculated (*number*)	Age (*hours*)	Mice that reached maturity (*number*)	Mice with parotid tumors (*number*)	Mice with adrenal tumors (*number*)	Sex of mice with tumors	Age at which tumors occurred (*months*)
7	15 min. centrif. at 3,000 r.p.m. filtered through collodion membrane (450 mμ porosity).	C3H	12	6–8	7	1 1	0 0	♂ ♀	9 10
15	15 min. centrif. 3,000 r.p.m. and 10 at 9,500 r.p.m. filtered through Morton glass filter.	C3H	10	6–8	3	0	0	—	—
17	Same as 15	C3H	10	6–8	3	0	1*	♂	12
18	Same as 15	C3Hf	16	4–6	3	1* —	— 1*	♀ ♂	11. 5 11. 5
24	15 min. centrif. at 3,000 r.p.m. and 10 at 9,500 r.p.m. Used as extract.	C3Hf	8	4–6	7	1* 1* 1 1 1 — —	1* 1* 1 — 1* 1* 1	♀† ♀‡ ♂♀ ♀ ♀ ♀§ ♂‖	9 9 10 10 12 14 12
33	Same as 24 except homogenate was frozen-thawed before centrifugation.	C3H	9	6–8	2	.....	1*	1♂	12
	Total	.....	65		25		9	9	

* Bilateral tumors.

† Mouse also had a mammary tumor and an ovarian tumor of same cell morphology as adrenal tumor.

‡ Mouse also had a mammary tumor and a small Harderian-gland tumor.

§ Mouse also had an ovarian tumor of same cell morphology as adrenal tumor.

‖ Mouse also had a fibrosarcoma in the subcutaneous tissue of the back.

they become moribund, they develop a granulocytosis with a predominance of myelocytes and the spleen becomes infiltrated with myeloid cells. This occurs even though there is no outward breakdown of the tumor. Anemia with an increase in reticulocytes and a drop in hemoglobin is not uncommon. Studies of the peripheral blood were made on all mice having parotid tumors that were sacrificed for study of the tumor.

Transplants of the parotid-gland tumors are difficult to grow. If the transplants are made into infant mice 1 to 4 days old they will grow slowly; the tumor develops at the site of inoculation in from 3 to 8 months. However, many of the infant mice inoculated with the tumor die within 12 to 21 days. The tumors will also grow when transplanted to 4- to 6-week-old mice that have been irradiated with 250 to 260 r [4] just prior to the inoculation. No metastases from the transplanted tumors have been seen. Marked histologic changes in the liver and adrenal cortex have been noted in irradiated mice bearing transplanted parotid tumors. This will be given in a later report.

Four different parotid tumors are being carried serially. One is in its 7th transplant generation and will now grow in 4 out of 5 untreated mice into which it is inoculated. The other three are still in their 2nd or 3rd transplant generation and will not grow in untreated mature mice. Several primary tumors which were transplanted to adult C3H mice, which had not been irradiated, failed to grow and two were lost after the first passage.

Histology

Parotid-gland tumors.—Microscopic sections of parotid-gland tumors from 17 mice of strain C3H and C3Hf were examined by Dr. Thelma Dunn, National Cancer Institute, who furnished the following description:

> "In the 17 tumors, the morphologic features were generally similar. All tumors appeared to arise in the parotid salivary gland. The tumors were generally multinodular (figs. 3 and 4). Considerable variation in morphology was observed in different nodules, so that the tumors were strikingly pleomorphic. Many of the nodules were composed of fusiform cells, which were often more ovoid and closely packed near the blood vessels. In others, the fusiform cells were frequently arranged in strands or cords, with intervening spaces (fig. 9). Other nodules showed a mixture of small tubules and acini of epithelial cells separated by fusiform mesenchymal cells (fig. 10). A frequent feature of nodules composed of epithelial cells with an acinar or tubular arrangement was the deposition of an amorphous hyaline substance between the epithelial elements (fig. 7). Cystic spaces were common, usually near the center of the nodules (fig. 8). The tumors, therefore, may be diagnosed as multinodular, pleomorphic tumors of the parotid gland, showing great variation in the morphology of different areas."

[4] Physical factors of irradation were: total-body irradiation, one tube; 250 KVP, 30 Ma.; beam filtration, 1.0 mm. Cu + 1.0 Al; target-mouse distance for tube, 50 cm.; dose rate 75 r per min.

Adrenal-gland tumors.—Many of these tumors have occurred bilaterally and have ranged from microscopic size to 25 mm. in diameter. The uncut tumors ranged from a pale pink to a deep hemorrhagic color and had a glistening appearance with a smooth surface. The larger ones were nodular. When cut they were very soft, and a finely minced tumor had the consistency of a thick fluid. Figures 11 to 16 show both gross and microscopic appearance of the adrenal tumors.

In mice bearing the tumor, the medulla and cortex of the adrenal gland were generally completely replaced by tumor cells. In the early neoplasms the tumor cells were seen in the process of infiltrating the cortex and, in some instances, were confined to the medulla.

The histology of these tumors was also studied by Dr. Thelma Dunn, who gave the following report:

> "Primary tumors from 9 C3H and C3Hf mice were examined. The tumors were composed of deeply basophilic cells, round to oval in shape, with relatively little cytoplasm, and round or slightly oval hyperchromatic nuclei (figs. 14 and 15). The cells were arranged in clumps separated by thin bands of connective tissue. Necrosis was frequent in the center of larger masses, and there was considerable central necrosis in the larger tumors. One tumor appeared more anaplastic, the cells were more fusiform, and invaded the upper pole of the kidney. One tumor showed an admixture of the usual basophilic cell, and groups of paler, more eosinophilic cells. One small tumor was surrounded by a rim of cortical tissue. Three tumors showed metastases to the liver (fig. 16). In 2 mice, tissue similar to the adrenal tumors was also found in the medulla of the ovary.
>
> "It is not possible with the present material to determine whether these tumors are medullary or cortical. They are unlike the medullary tumors described by Jones and Woodward (*15*), since the cytoplasm is heavily basophilic and the nuclei do not show polarization. They more closely resemble cortical tumors which follow castration in some strains of mice, although in the present material the smaller tumors appeared to start within the medulla. They should be designated for the present no more specifically than as 'tumors of the adrenal gland'."

Metastases from the primary spontaneous adrenal tumors have been observed in the following organs: liver, lungs, pancreas, peribronchial lymph nodes, peripheral lymph nodes, and possibly the ovaries. (The same type of tumor cells were observed in the ovaries in two instances; the possibility exists that these may have been primary tumors in the ovaries.)

Subcutaneous transplants of the adrenal tumor kill C3H mice in from 8 to 12 weeks. Three different tumors are being carried serially and are now in from the 7th to the 21st transplant generation. Irradiation of the mice (250 r) prior to the transplanting of the tumors was carried out for the first few generations, but now the tumors grow progressively in 100 percent of the mice inoculated. Metastases to the ovary, and to

peripheral lymph nodes, of an adrenal tumor transplanted subcutaneously are shown in figures 17 and 18.

Extracts from Normal C3H Tissues Inoculated into Newborn C3H Mice

Extracts were prepared from normal tissues of strain C3H mice 4 to 6 weeks of age, using the same technique for extracting as was used with the leukemic tissues, except that the different organs were extracted separately. Using spleen, liver, kidney, and thymus, several of the same organs from different mice were pooled, homogenized, extracted, and centrifuged at 3,000 r.p.m. for 15 minutes at 0° C.; 0.1 ml. of the supernatant was inoculated intraperitoneally or subcutaneously into newborn C3H mice. A total of 30 mice were observed for a period of 12 to 16 months, and none developed leukemia, parotid- or adrenal-gland tumors.

Discussion

"Since cancer, on the one hand, develops with great frequency in selective tissues but may occur in many of them, the cause must be highly specific yet must also have the property of infinite variation," [Duran-Reynals (*16*)]. Specificity of the virus for certain cells in one strain or species of a host, and the capacity for variation of the virus in other cells of the same or different species, are well-known characteristics of viruses.

In the experiments presented, 3 of possibly 4 different neoplasms have been observed as a result of inoculating mice with cell-free material prepared from leukemic tissues of mice. Since only a few leukemias were observed in the experimental strain C3H mice it may be assumed that this strain of mice, when exposed to leukemic extracts or filtrates under the conditions of the experiments, are only slightly susceptible to the leukemogenic activity reported by Dr. Gross for such cell-free extracts.

The unusual parotid- and adrenal-gland tumors, which have not been observed in control animals of the C3H strain but only in those mice inoculated shortly after birth with leukemic extracts or filtrates, indicate the potential variations of the tumor-producing activity of the cell-free leukemic extracts or filtrates. A similar variation in activity of tumor-producing viruses has been demonstrated. Duran-Reynals (*17–19*) has demonstrated that the virus which produces the Rous sarcoma, under certain conditions causes a rapidly fatal hemorrhagic disease, and under other conditions can produce a variety of tumors ranging from lymphosarcomas to muscle tumors. Furth (*20*) found that lymphomatosis, myelomatosis, and endothelioma in chickens were caused by the same filterable agent. Rose and Rose (*21*) have shown the multiplicity of action of the Lucké tumor agent, the expression of which depends on the host inoculated.

In the present studies the multiple neoplasms arising in mice inoculated with extracts or filtrates from leukemic-mouse tissues could be explained as being due to a virus, the tumor produced depending on the susceptibility of the host. The possibility of more than one virus must

be considered, yet it is of interest to note that 5 out of 9 mice that developed neoplasms of the adrenal glands also had parotid-gland neoplasms, and 2 had ovarian tumors (table 4), all of which occurred simultaneously as primary neoplasms. It would thus be necessary to postulate the existence of 3 viruses in the leukemic extract, each having the potentialities of producing tumors in the same period of time. The question may be raised as to the presence of a toxic substance or a carcinogen in the leukemic extracts. This can be settled only by the isolation of the active principle.

Summary

1) Tumors of the parotid gland were observed in some of the C3H and C3Hf mice inoculated shortly after birth with extracts or filtrates prepared from AKR and C3H leukemias. Those receiving extracts and filtrates from C3H leukemia #19, when 4 to 8 hours old, had an incidence of 36 percent parotid-gland tumor.

2) Tumors of the adrenal gland were produced in C3H and C3Hf mice inoculated when newborn (4 to 8 hours old) with extracts or filtrates from C3H leukemia #19. The majority, 5 out of 9, of the mice with these tumors also had parotid-gland tumors. An incidence of 36 percent of adrenal-gland tumors was observed in this group.

3) An incidence of 2.6 percent of leukemia was produced in 3-month-old strain C3H mice following the technique described by Gross. This is within the normal incidence for the strain. Generalized leukemias were also produced in a few adult mice 10 to 14 months after inoculation in the anterior chamber of the eye with embryonic-tissue transplants that had been exposed to leukemic extracts.

4) Extracts from normal C3H tissues, when inoculated into normal C3H mice, in no instance produced either parotid-gland tumors, adrenal-gland tumors, or leukemia.

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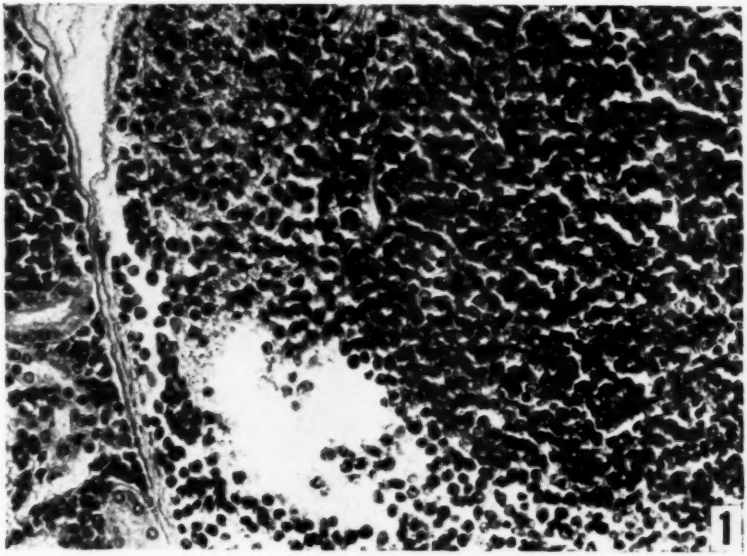
PLATE 93

FIGURE 1.—C3H male bearing a subcutaneous transplant of leukemia #19 in its 50th transplant generation. Infiltration of renal lymph node and kidney by leukemic cells. × 300

FIGURE 2.—Same mouse as in figure 1, showing infiltration of leukemic cells into the liver. × 300



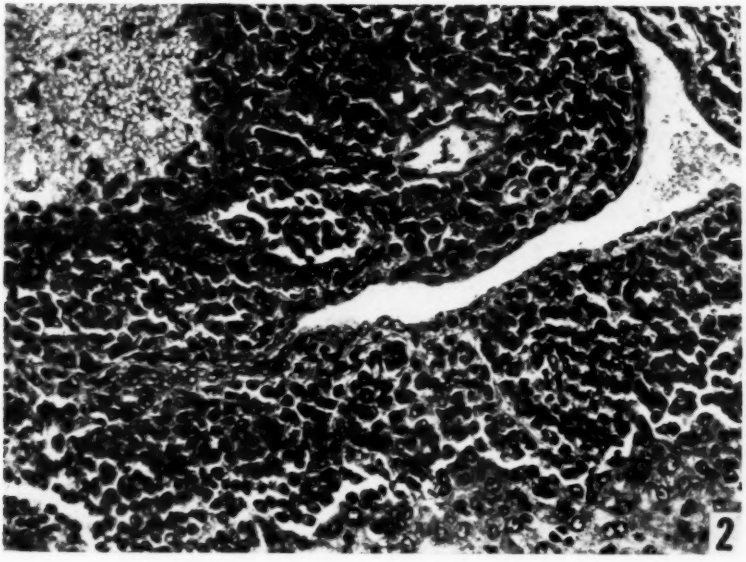


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PLATE 94

Parotid-gland neoplasms in strain C3H and C3Hf mice inoculated shortly after birth with leukemic mouse tissues

FIGURE 3.—C3H male inoculated with extract of leukemia RIL6. Bilateral parotid-gland tumors when 5 months old. Tumors appearing principally in lower neck region.

FIGURE 4.—C3H male inoculated with extract of leukemia #19. Bilateral parotid-gland tumors when 10 months old. Tumors appearing principally in parotid-gland region.

FIGURE 5.—C3Hf female inoculated with filtrate of leukemia #19. Died at 10 months of pneumonia. Mammary tumor in inguinal area. Early parotid tumor showing nodular formation.

FIGURE 6.—C3Hf male inoculated with extract of leukemia #19. Advanced bilateral parotid-gland tumors showing nodular clusterlike formations of the tumor. Enlarged abdomen due to hemorrhagic intraperitoneal fluid; mouse found to have bilateral adrenal tumor when abdominal wall was opened.

Stewart

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PLATE 95

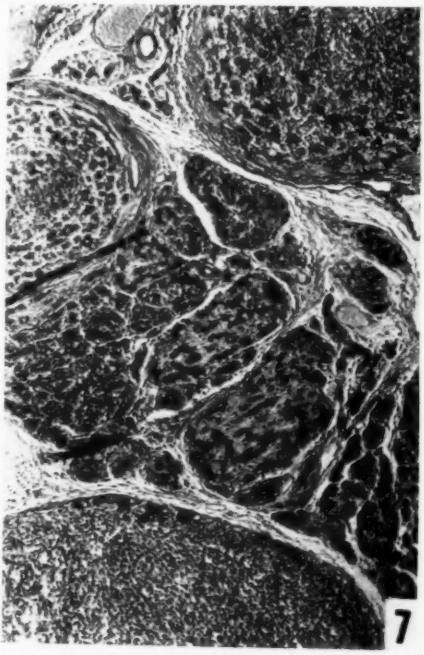
Parotid-gland neoplasms

FIGURE 7.—Section from tumor shown in figure 3, plate 94. Note variation in structure of nodules. Upper *right* and *middle* portions show nodules composed of acini of epithelial cells separated by fusiform mesenchymal cells. Amorphous hyaline substance is seen between the epithelial elements. *Lower* portion is composed of fusiform cells only. *Middle right* area shows relatively normal parotid gland tissue. × 85

FIGURE 8.—Section of a parotid tumor showing a cystic space. × 230

FIGURE 9.—Tumor from C3Hf female having bilateral parotid-gland tumors. Sacrificed at 9 months of age. Section shows fusiform cells arranged in strands or cords with intervening spaces. × 80

FIGURE 10.—Tumor from C3H male, sacrificed at 9 months. Section shows tumor with acini of epithelial cells separated by fusiform mesenchymal cells. × 450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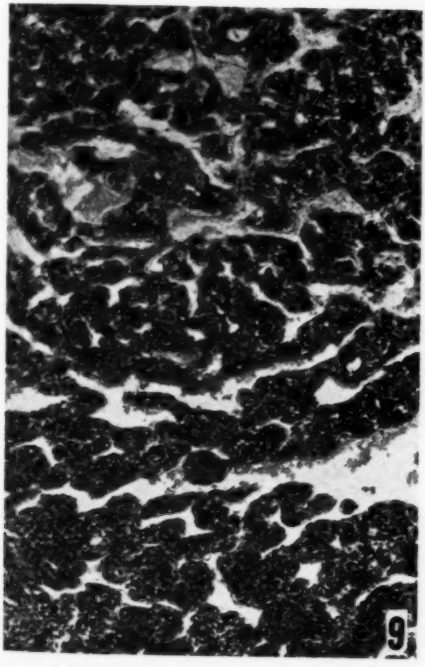
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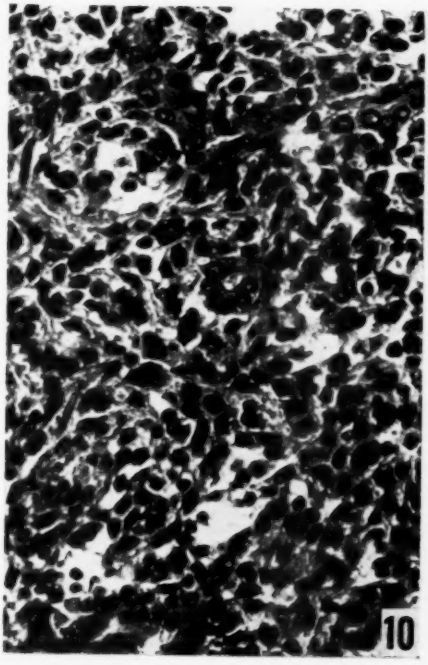
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PLATE 96

Adrenal-gland neoplasms in C3H and C3Hf mice inoculated shortly after birth with leukemic mouse tissue

FIGURE 11.—C3Hf female inoculated with extract of leukemia #19. Sacrificed when 10 months old. Bilateral adrenal tumors. Note nodular nature of large tumor on right. Histologic study of tissues also showed early bilateral parotid tumors.

FIGURE 12.—C3H male inoculated with filtrate of leukemia #19. Died when 11.5 months old. Bilateral adrenal tumors. Note tumor on *right* is hemorrhagic and on *left* it is pale and glistening.

FIGURE 13.—Adrenal gland from C3H male inoculated when newborn with filtrate of leukemia #19. Sacrificed when 10 months old. Unilateral adrenal tumor and early unilateral parotid tumor. Section shows adrenal gland in which the central portion is occupied by a cellular tumor mass surrounded by atrophic cortical adrenal cells with an intervening zone of marked congestion. × 85

FIGURE 14.—Adrenal tumor from C3H female inoculated when newborn with filtrate of leukemia #19. Sacrificed when 9 months old. Bilateral adrenal and parotid tumors. Section of adrenal tumor. × 100

FIGURE 15.—High-power magnification of adrenal tumor from tumor in figure 14. × 515

FIGURE 16.—Adrenal tumor metastasis into liver from mouse shown in figure 11. × 450

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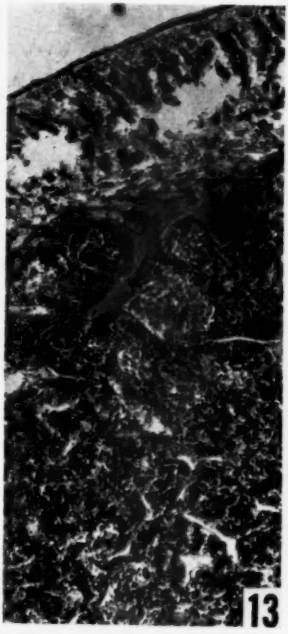
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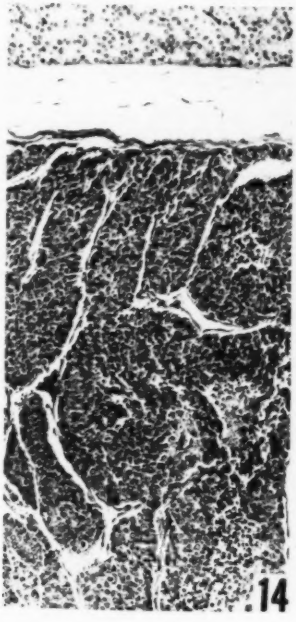
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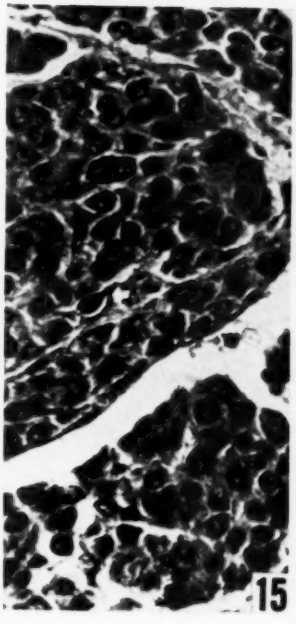
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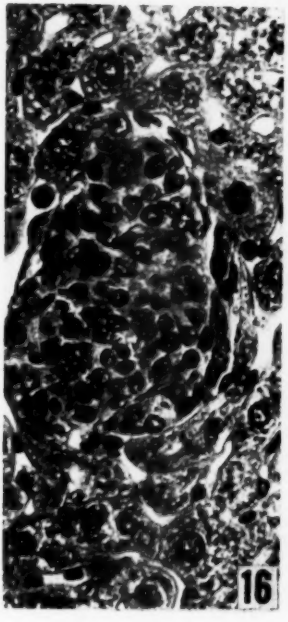
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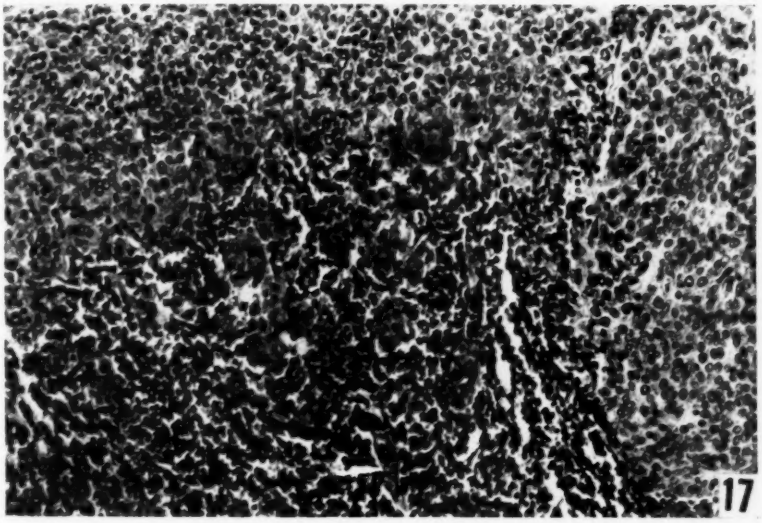
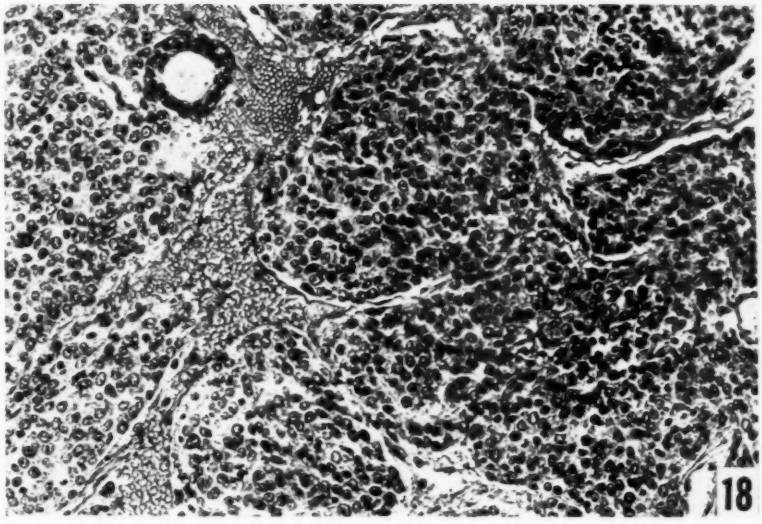
PLATE 97

FIGURE 17.—Adrenal tumor metastasis into peripheral lymph node. × 67

FIGURE 18.—Adrenal tumor metastasis into ovary. × 85





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